University of Alberta

Transformations of Carbon in Hydrocarbon-Contaminated Soils

by

Vanessa Laurelle Bailey

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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ABSTRACT

Petroleum-derived hydrocarbons contribute to the available C supply for microorganisms in contaminated soils. This C may be metabolized and transformed by the soil microorganisms, producing new biomass, CO_2, or partially transformed metabolites. Carbon-14 labeled model organic contaminants (MOCs) were used to describe these transformations in two aged contaminated soil samples. The MOC for the creosote-contaminated soil was pyrene; for the oil-contaminated soil, octadecane. Collection of the ^{14}C as $^{14}CO_2$ and its redistribution into humic-C indicated that these are important fates of the contaminant. Both soils had mineralized 50% of the applied ^{14}C to $^{14}CO_2$ within the first 3 months of a 12-month incubation. A maximum of 32% of the applied ^{14}C was recovered in the humic-C of the oil-contaminated soil during the incubation. These transformations may be reduced through sequestration of the MOC into a non-aqueous phase liquid (NAPL), which may be the form in which the contaminant is present. The addition of dichloromethane to these soils reduced the degree to which the contaminants were transformed such that the quantity of $^{14}CO_2$ evolved decreased by 50% in the oil-contaminated soil and by 98% in the creosote-contaminated soil. As well, enrichment of the humic C with ^{14}C was decreased to 5%, indicating reduced biotransformation of the MOC. The soils did not appear to be sterilized by the solvent, as the metabolism of other ^{14}C-labeled compounds (glucose, cellulose, or acetate) added to these soils following solvent treatment was observed to be similar to that observed in the absence of solvent treatment. This suggested that the NAPL form of the contaminant was non-uniformly distributed in these two soils and was redistributed by the solvent. When the non-type MOC was applied to either soil, the indigenous microorganisms appeared to be limited in

their ability to mineralize it, evolving <3% of the applied ^{14}C to $^{14}CO_2$ during the incubation, although some transformations within the soil C fractions were observed. In properly managed systems, contaminant-derived C may not be a great obstacle to bioremediation; the compounds may simply be additional substrate for the indigenous microbial populations. These compounds additionally have the potential to substantially enrich the soil organic matter with their C.

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List of Abbreviations

BC	Biomass-carbon
CUE	Carbon utilization efficiency
DEOC	Dichloromethane extractable-carbon
DNAPL	Dense nonaqueous phase liquid
DOC	Dissolved organic carbon
EC	Electrical conductivity
ESTAC	Environmental Science and Technology Alliance of Canada
HC	Humic-carbon
HNC	Humin-carbon
K_d	Partition coefficient
K_{oc}	Organic carbon normalized partition coefficient
K_{ow}	Octanol-water partition coefficient
LUST	Leaking underground storage tank
MGP	Manufactured gas plant
MOC	Model organic contaminant
MRT	Mean residence time
MWD	Mean weight diameter
NAPL	Nonaqueous phase liquid
OM	Organic matter
PAH	Polycyclic aromatic hydrocarbon
PCP	Pentachlorophenol
PI	Polarity index
SOM	Soil organic matter
TNT	Trinitrotoluene
WHC	Water holding capacity
WSA	Water stable aggregates
WSC	Water soluble-carbon

CHAPTER 1

INTRODUCTION

Contamination of soils by organic contaminants is a serious problem. A popular treatment for contaminated soils is bioremediation. Bioremediation is "the use of microorganisms to degrade pollutants and restore environmental quality" (Atlas and Cerniglia 1995). The process of bioremediation is often distilled down to the simple, ideal formula (Atlas and Cerniglia 1995, Bossert and Compeau 1995):

$$Organic\ Contaminant \xrightarrow{\text{capable microorganisms}} CO_2 + H_2O + biomass$$

In addition, the transformation may produce partially oxidized metabolites that may be subject to reactions within the soil. This literature review focuses on organic contaminants and their transformations in soil.

Soil Contamination

Causes of contamination

Organic contaminants may enter the soil through many different pathways. Accidental spills, poorly designed hazardous waste facilities (Pavlostathis and Mathavan 1992), and leaking underground storage tanks (LUSTs; Atlas and Cerniglia 1995) are all routes by which organic contaminants may enter soils. The magnitude of the problem is difficult to assess. One estimate is that there are >250 000 LUSTs at service stations across the United States (Atlas and Cerniglia 1995).

Manufactured gas plants (MGPs) are often singled out as a category of contaminated sites (Peters and Luthy 1993, Ramaswami and Luthy 1997, Yeom *et al.*, 1996). These sites were usually in operation for several decades, and upon their decommissioning, soil contamination with coal tar and petroleum residues is identified. It is suspected that there are over 1000 contaminated MGP sites in the United States alone (Peters and Luthy 1993). Wood preserving facilities are also likely sites of contamination, after many years of using chemicals to treat lumber (Deschênes *et al.* 1996), and Mueller *et al.* (1989) estimate the number of active and inactive such sites in the United States to be 700.

Statistics for contamination events in Alberta are unknown. The Alberta Environmental Protection Agency does not keep a count of reported contamination events due to the range of events considered "contamination." Reports of contamination are required for events ranging from small gasoline spills at the scene of a car accident, through excavation of tanks at service stations, to sites of massive oil well blowouts.

Soils at industrial sites may be contaminated with a variety of pollutants and the date or duration of the contamination is usually uncertain. Due to long life span of such sites, it is reasonable to believe that much of the contamination is cumulative, the result of many years of site use.

Types of contamination

A rich literature exists concerning pesticide, inorganic compound, and metal contamination of soils, however this thesis examines hydrocarbons as contaminants, *i.e.*, as materials that are present where they are not wanted. The two main sources of hydrocarbon contamination in soils are creosote and petroleum oils.

Coal tar, or creosote, exists as a nonaqueous phase liquid (NAPL) that is denser than water and contains high concentrations of polycyclic aromatic hydrocarbons (PAHs; Mueller *et al.* 1989, Peters and Luthy 1993, Ramaswami and Luthy 1997). Creosote is an oily compound containing a wide variety of PAHs. Other components of creosote include oils and greases with asphaltenes, polar compounds, saturated organic compounds, (straight- and branched-chain alkanes of various lengths and various isoprenoid compounds of various length, complexity, and recalcitrance), and chlorinated organic compounds (Deschênes *et al.* 1996, PACE 1990, Peters and Luthy 1993). PAHs are compounds made of conjoined aromatic rings and include representatives varying from 2-member rings such as naphthalene, to 4, 5, and 6-member rings such as pyrene and chrysene (4 rings; Liu *et al.* 1992, Song *et al.* 1990). PAHs are of particular concern in the environment because many are known to be, or suspected to be, carcinogenic or teratogenic (Cerniglia 1992, Kawahara *et al.* 1995, Liu *et al.* 1992, van Schooten *et al.* 1995, Wilson and Jones 1993).

Petroleum contains a range of compounds classified among four proximate categories: asphaltenes, paraffins, aromatics, and resins (Petrakis *et al.* 1980, Launen *et al.* 1995). Included within these categories are aliphatic compounds: straight and branched chain alkanes, cycloalkanes, and alkenes; aromatics: monoaromatics and PAHs and resins or NSO-containing compounds (McGill *et al.* 1981). The asphaltenes are high molecular weight, are soluble in toluene and precipitated in pentane, and may be highly condensed aromatic and cyclic polymers containing N, S, and O (McGill *et al.* 1981).

The conditions under which these contaminants exist in soil further compound their hazard because they occur as a multiphase problem: the solid phase of the soil, soil solution, and soil vapour are all contaminated. If present as a free phase, the contaminant NAPL will modify the normal attributes of the soil (Pollard *et al.* 1994), and therefore modify the predicted sorption behaviour (bioavailability) of the contaminants. Specific contaminant compounds that are highly hydrophobic may sorb to the NAPL, and be inaccessible to microorganisms capable of metabolizing them.

Bioavailability of contamination

Microbial access to contaminants (an aspect of bioavailability) may be the major limitation to successful bioremediation. The potential substrate, the contaminant, may be made unavailable by a number of mechanisms: it may be physically sorbed to native soil constituents (Deschênes *et al.* 1996, Murphy *et al.* 1990, Pavlostathis and Mathavan 1992), it may be sorbed in the NAPL component of the contamination (Efroymsen and Alexander 1995, McGroddy *et al.* 1996, Yeom *et al.* 1996), or it may be protected within small pores in aggregates from which microorganisms are excluded by size (Ramaswami and Luthy 1997). Several opinions of how microbes metabolize substrates exist: the substrate may have to be in aqueous solution, the microbe may be able to colonize the surface of a non-aqueous phase to access the substrate, or proximity may be a factor.

Sorption of the contaminant to soil constituents may be described mathematically. Models that are commonly used to describe types of sorption include the Langmuir model and the Freundlich model. If the mechanism of sorption is known to be partitioning, such as may occur when hydrophobic compounds interact with a free NAPL or SOM, the partition coefficient (K_d) relates the solution concentration (C) of a compound to the fraction sorbed (S) to a solid surface (Scow and Hutson, 1992):

$$S = K_d C \qquad \text{[Eq. 1-1]}$$

The K_d may be normalized to the soil organic carbon content (f_{oc}) and a new constant, K_{oc} is derived (Xing *et al.* 1994):

$$K_{oc} = \frac{K_d}{f_{oc}} \qquad \text{[Eq. 1-2]}$$

The K_{ow} is the ratio of the solubility of a compound in octanol to its solubility in water. It can be used to estimate the degree to which a hydrophobic organic compound will partition into organic matter or a contaminant NAPL in soil. The K_{oc} may be calculated from the K_{ow}, by solving linear equations (Xing *et al.* 1994):

$$\log K_{oc} = a + b \log K_{ow} \qquad \text{[Eq. 1-3]}$$

In Eq. 1-3, a and b are empirical constants.

The normalization of K_d to K_{oc} is useful because K_{oc} is compound specific, whereas K_d is soil-and-compound specific. A single organic contaminant may have different partition coefficients in different soils (Murphy *et al.* 1990), a phenomenon that was observed in studying contaminants in field-contaminated soils (Pavlostathis and Mathavan 1992). The values of K_d for hydrophobic organic contaminants vary as the soil organic C contents vary. Another implication of this normalization is that the contaminant is being sorbed to soil organic matter components. Xing *et al.* (1994a), sorbed benzene, toluene, and xylene to a range of biopolymers and humic acid in pure systems. As these and similar compounds are present in soil, it is likely that organic chemicals will sorb to such materials. Xing *et al.* (1994b) reported that K_{oc} decreased with increasing mass ratio [(O+N)/C] of non-protein organic sorbents. They proposed the term "polarity index" (PI) for this mass ratio, recognizing it ignores polarity that may arise from configuration and structure. The advantage of K_{oc} as a chemical-specific variable, may be illusory if the native organic phase varies substantially among soils and sediments; or if the native organic phase is dominated by co-contaminants or NAPLs that become sorbent phases. Consequently, the assumption of uniformity of sorbents in Eq.1-2 may lead to errors in predictions of sorption by soils and in retardation coefficients used in transport and fate models.

Murphy *et al.* (1990) examined the interactions of three organic chemicals: carbazole, dibenzothiophene, and anthracene with aquifer sediments. They concluded that the organic compounds had sorbed to mineral-bound humic materials. Pyrene has been observed to bind more strongly to sediments than its log K_{ow} (5.32; Sims and Overcash 1983) predicts (McGroddy *et al.* 1996). The authors hypothesize that this is due to interactions between the pyrene, the soot and diesel of the PAH carrier matrix and organic matter.

Aging of contaminants in soils

Contaminated soils are often ignored or left untreated until legislation forces action or a suitable treatment strategy can be devised. Consequently, the contaminant may "age" in the soil, resulting in a contaminated soil that has unique characteristics that cannot be easily simulated for laboratory studies.

Some of the aging reactions may be: evaporation, photolysis, hydrolysis, and biotransformation. The residual mix may be less volatile, and has higher K_{oc} values than the original contaminant (Pollard *et al.* 1994) and may therefore be more persistent.

Aged field-contaminated soils have been examined both to clarify the behaviour of the aged contaminants (Pavlostathis and Mathavan 1992, Yeom *et al.* 1996) and to devise more realistic model systems (Deschênes *et al.* 1996). Both Pavlostathis and Mathavan (1992) and Yeom *et al.* (1996) found that desorption of contaminants from soil components and contaminant NAPLs was the primary limitation to successful degradation. Deschênes *et al.* (1996) used a weathered (20 yr) creosote-contaminated soil to examine the efficacy of surfactants at facilitating biodegradation, studying both the metabolism of PAH components of the contaminant mix and the fate of ^{14}C-phenanthrene applied to this soil. Hatzinger and Alexander (1995) attempted to study weathering of chemicals in soils in the lab by aging ^{14}C-phenanthrene in three soils (a muck, a loam, and an aquifer sand) for 300 d. Aging did not change the mineralization rates of the phenanthrene in the sand, but resulted in much lower phenanthrene mineralization rates in the muck and the loam. The extractability of phenanthrene in *n*-butanol was diminished from 96% to 67% in the muck after aging. The extractability was also reduced in the loam after aging. They speculated that both slow desorption of phenanthrene from humic materials or physical occlusion within micropores and aggregates were responsible for the reduced extractability. Enhanced mineralization of the phenanthrene following disruption of the aggregates by sonication is consitent with the latter hypothesis.

Bioremediation

Bioremediation is one treatment option for contaminated soils that has become very popular. Several technologies and strategies have been proposed that take advantage of natural microbial processes.

Strategies and technologies of bioremediation

Bioremediation plans should be site-specific, tailored to specific contaminant-soil-climate combinations (Hicks and Caplan 1993). There are four categories of land treatment options: *in situ*, enhanced land treatment, slurry bioreactor, and bioventing (Pollard *et al.* 1994). The last category is limited to volatile components in aerobic systems.

In situ strategies treat the soil on site, with no excavation. Treatment may be similar to enhanced land treatment options, with respect to amendments and tilling. *In situ* treatment is the least intrusive option, and requires the fewest inputs. However, not all soils are suited for this treatment. Madsen (1991) suggests a generalized "plan-of-attack" to determine if a soil is suited for *in situ* treatment:

1. Conduct a preliminary site investigation to assess the field, the type of contamination, and the capabilities of the indigenous microorganisms.

2. Assess the potential for *in situ* biodegradation by looking for aerobic and anaerobic zones and zones of varying contaminant concentration (toxicity).
3. Conduct a detailed site characterization.
4. Seek proof of *in situ* biodegradation by looking for metabolites of known contaminants in laboratory studies.

Subsurface contamination is unlikely to be remediated rapidly by *in situ* treatment, because O_2 and microorganisms become more limiting with increasing depth. Climate conditions at the site may also hinder *in situ* bioremediation (Pollard *et al.* 1994).

Slurry bioreactors require large inputs of energy, not the least of which is the need to excavate all of the contaminated soil. These systems supply O_2 and nutrients to contaminated soils that are constantly being agitated usually in a rotating drum bioreactors (Gray *et al.* 1994, Kruger *et al.* 1995). These systems may also include seeding of the soil slurry with microorganisms known to metabolize the contaminants present. This treatment option is attractive because it is quick and suitable for cleaning contaminated subsurface soils in which O_2, nutrients, and microorganisms may be limited.

Enhanced land treatment includes landfarming, biopiling, and composting. These treatments usually involve amending the soil with nutrients, moisture, and sometimes microorganisms (Pollard *et al.* 1994, Rosenberg *et al.* 1992, Shaw *et al.* 1995). The soil may be excavated and moved to a lined site to contain leachates, or piled and amended with co-substrates, and may be aerated by frequent tilling or windrowing. The underlying principle of these options is that soils may serve as "solid state bioreactors" (Bossert and Compeau 1995).

Phytoremediation is another treatment option that has been proposed (Aprill and Sims 1990, Reilley *et al.* 1996, Shimp *et al.* 1993). This strategy is simply to include plants in passive bioremediation systems to take advantage of the increased numbers of microorganisms and co-substrates in the rhizosphere. Cunningham and Ow (1996) include reference to phytoremediation in the direct sense: plants may serve as sinks for contaminants.

Processes of bioremediation

The general objective of bioremediation, as stated earlier, is the microbially mediated metabolism of organic compounds to water, CO_2, and biomass. Indeed, no examination of bioremediation can ignore the mineralization of the contaminant to CO_2, and often this is the main indicator of bioremediation success (Hatzinger and Alexander 1995, in der Wiesche *et al.* 1996, Walton *et al.* 1989). However, the metabolism of contaminants may produce stable metabolites, or metabolites that may be stabilized by soil and these merit examination. Organisms capable of metabolizing hydrocarbons are believed to be ubiquitous and have been isolated from most soils and sediments, not just those that have been contaminated (Rosenberg 1991, Venkateswaran and Harayama 1995).

Hydrocarbon metabolism commonly requires a suite of microorganisms working syntropically (Herman *et al.* 1994, Gray *et al.* 1994). Many studies have found that summing the degradative abilities of individual species in a consortium of microorganisms does not equal the capabilities of the mixed culture (Trzesicka-Mlynarz and Ward 1995, Venkateswaran and Harayama 1995, Wolfaardt *et al.* 1994). Cometabolism of more

complex hydrocarbons such as benzo[a]pyrene (Kanaly *et al.* 1997) and asphaltenes (Rontani *et al.* 1985) may also be an important metabolic process. Bossert and Compeau (1995) define cometabolism as occurring when: "a substrate is only partially metabolized in the presence of other substrates (cosubstrates) and cannot itself provide C for energy or growth to microorganisms." Atlas and Atlas (1991) note that organisms capable of metabolizing aromatic substrates are likely distinct from those that metabolize aliphatics.

The processes by which hydrocarbons may be transformed or attenuated (removed from interacting with the environment) in the environment are many: volatilization, photooxidation, chemical oxidation, bioaccumulation, partitioning to soil components, leaching, and microbial degradation (Bossert and Compeau 1995, Cerniglia 1992). For PAHs, the rate of biodegradation is usually inversely proportional to the number of rings constituting the PAH; the lower molecular weight PAHs are usually the most rapidly degraded (Cerniglia 1992). It is widely accepted that the first step of PAH metabolism is oxidation (Fig. 1-1; Cerniglia 1992, Launen *et al.* 1995, Trzesicka-Mlynarz and Ward 1995). Generic oxygenases usually mediate this oxidation. Fungi accomplish this via a monooxygenase that leads to a *trans*-dihydrodiol and eventually to catechol (Atlas and Cerniglia 1995, Launen *et al.* 1995). The *trans*-dihydrodiol may also lead to epoxides, dead-end metabolites, or DNA adducts. Bacteria mediate the initial oxidation with a dioxygenase that produces *cis*-dihydrodiols followed by dehydrogenation reaction (Atlas and Cerniglia 1995). Three of the most common convergence points in PAH metabolism are catechol, gentisic acid and protocatechuic acid, single-ring aromatic compounds with alcohol or carboxylic acid moieties attached (Wilson and Jones 1993). Partly oxidized intermediates may serve as reactive sites for association with soil components (Bossert and Compeau 1995).

White rot fungi and the enzymes they produce are able to metabolize PAHs (Andersson and Henrysson 1996, in der Wiesche *et al.* 1996, Vazquez-Duhalt *et al.* 1994). Andersson and Henrysson noted that "dead-end" metabolites (diones of the substrates) accumulated when anthracene, benz[a]anthracene and dibenz[a,h]anthracene were incubated with each of five species of white rot fungi in soil systems. These metabolites may become future components of soil organic materials.

Lower molecular weight aliphatics ($<C_9$) are lost primarily to volatilization (Song *et al.* 1990). The high boiling range distillates are usually slow to degrade (Song *et al.* 1990, Wang and Bartha 1990), although a *Pseudomonas* sp. capable of metabolizing resins as the sole source of C and energy has recently been isolated (Venkateswaran *et al.* 1995). Biodegradation of aliphatic hydrocarbons, as with the PAHs, is usually via an initial oxidation reaction (Bossert and Compeau 1995, Herman *et al.* 1993). Metabolism of aliphatic chains via β-oxidation (Fig. 1-2) results in the release of C_2 units (acetate units) that are quickly metabolized.

Phytoremediation is simply the enhancement of the above reactions in the rhizosphere. The rhizosphere is host to increased numbers of microorganisms (Shimp *et al.* 1993) and simple exudates that may serve as cosubstrates or primers to stimulate the activity of the rhizosphere microorganisms (Hsu and Bartha 1979, Shimp *et al.* 1993). Potential plant uptake of hydrocarbons is closely related to the properties of the chemical. Direct uptake and accumulation is possible for compounds with low K_{ow} ($\log K_{ow} = 0.5\text{-}3$). Hydrophobic compounds ($\log K_{ow} > 3.0$) are too strongly bound to root surfaces to be

taken up. According to Schnoor *et al.* (1995) compounds that are very soluble (log K_{ow} <0.5) may not sorb enough to be transported through plant membranes.

Soil C Dynamics

In considering the processes of bioremediation and their possible end points, it is useful to note that when McGill (1975) modeled oil decomposition in soil to predict N-demand, humification was included. The cycling of contaminant-C into the native soil organic matter (SOM) is an optimistic fate of that C.

Mineralization of C in soil

Carbon mineralization to CO_2 removes C from the soil system. Although plants and some microorganisms fix CO_2 and return C to soils as residues, this review will focus on the aspects of C dynamics that keep C in the soil.

Soil C fractions

Soil C may be described in several ways: chemically, by its constituents (carbohydrates, proteins, *etc.*); physically, by density separations; biologically, by biomass and its products, or; kinetically, as being composed of active and resistant fractions. I have chosen to study soil C in this thesis from a biochemical perspective, examining the biologically mediated transformations of contaminant-C, beginning with the contaminant-C fraction. A wide variety of solvents have been used to describe contaminant-C in soil. I have elected to describe this fraction as the dichloromethane-extractable organic materials, as dichloromethane is a commonly used solvent for extracting both total petroleum hydrocarbons and PAHs (Deschênes *et al.* 1996, Kanaly *et al.* 1997, Liu *et al.* 1992, and Song *et al.* 1990).

SOM can be described as being made up of several classically defined soil C fractions: biomass, humic materials, water soluble C, non-extractable C (humin), and in contaminated soils, a contaminant-C fraction (DEO-C). SOM can also be described as "a complex mixture of humified and non-humified residues" (Andreux *et al.* 1990). Other researchers have fractionated SOM into humin, humic and fulvic acids, light and heavy fractions, and particulate-associated C (Wander and Traina 1996) or simply into mineral-associated size fractions (Cambardella and Elliot 1994).

The turnover rate of SOM is difficult to determine because the composite fractions have independent turnover times. A convenient tool for estimating turnover of soil C takes advantage of the characteristic isotope signatures of C3 (Calvin cycle) and C4 (Hatch-Slack cycle) plants. Plants that incorporate C through the Calvin cycle are enriched with ^{13}C relative to those that use the Hatch-Slack cycle. The $\delta^{13}C$ value for C3 plants varies between -22 and -34‰ and for C4 plants the range is -6 to -20‰ (Bender 1971; Smith and Epstein 1971). C3 and C4 plants grown under the same environmental conditions exhibit an average difference of 12-14‰ in their $\delta^{13}C$ values (Bender 1971). Consequently switching a soil with a history of C3 grasses to maize (C4) for a period of time "labels" the recent SOM by decreased ^{13}C contents. In this manner, 22% of the OM in a silty clay soil was calculated to have turned over in 13 yr of cropping with maize and 19% of the OM had turned over in a clay loam soil in 23 yr (Balesdent and Mariotti 1987). In this study, the youngest OM was associated with clay-size particles, and the oldest OM

was highly aromatic and associated with silts. The time periods are not, however, good indicators of the turnover rate of the whole SOM because the fraction of SOM enriched is more dynamic than is the whole. Using soils labeled with ^{14}C-glucose as a tracer, Ladd *et al.* (1992) found that the biomass C was much more highly labeled than the whole soil. As the labeled biomass turned over, measured rates of C mineralization slowed down, presumably in response to the increasing complexity of the C forms metabolized and protection of the biomass within soil aggregates.

Cambardella and Elliot (1994) isolated SOM associated with soil particle size fractions and aggregates and identified a C fraction they denoted the "ELF" (enriched labile fraction). This fraction is believed to be transient OM when found on the surfaces of microaggregates. They speculated that ELF could be protected from microbial transformation if it becomes occluded within aggregates, and in which case it is probably an important component of the slow OM pool. This is significant, as the dynamics and protection described are similar to the mechanisms by which weathered contaminants may persist in soils, discussed earlier.

The evolution of radiocarbon-dating methods provided another isotope tracing mechanism for dating SOM: These methods take advantage of the natural ^{14}C activity of the C in soil fractions to compare their relative stabilities. This lead to the information that the humin fraction of SOM has the highest mean residence time (MRT) in soils, and that these values may be greater than 1000 yr (Campbell *et al.* 1967). The most labile components of SOM, such as some of the hydrolysates, have the shortest MRTs, and are therefore the youngest (25 yr). Martel and Paul (1974) used C-dating methods to compare the distribution of ^{14}C activity among SOM fractions with the distribution of ^{14}C-labeled material added to the soil in the laboratory. This research provided evidence that the light fraction (density fractionation of SOM) was the youngest. These younger fractions have the highest turnovers of soil C and will be the fraction most affected by soil C additions.

Water soluble C (WSC) is a simple, yet meaningful component of SOM. Microbial metabolism often solubilizes complex C (Cogle *et al.* 1989, Jawson and Elliot 1986). During the course of wheat straw decomposition in a sand-nutrient mixture, Jawson and Elliot (1986) observed an initial decrease in WSC in the first few days, followed by increases for the rest of the study (192 d). The colour of the WSC darkened with time and it appeared that water-soluble humic materials were being derived from the decomposing straw. Cogle *et al.* (1989) amended soil with ^{14}C-labeled wheat straw and observed an initial increase in WSC until day 4, followed by a decrease. They identified WSC as the most readily decomposed form of soil C and noted that the amount of WS^{14}C never exceeded 2.6 % of the total ^{14}C added for the duration of the incubation (35 d). The inference drawn in the latter study suggests that as complex C is solubilized it becomes more bioavailable and is further metabolized. Contrasting these two studies suggests that the water-soluble humic materials produced in the artificial system may be incorporated into the native SOM.

Except when cometabolism occurs, all metabolic processes in soil should be reflected in the biomass. The biomass mediates the biotransformations in soil so it is a useful "window" to activities in the soil and the isotopic composition of the biomass responds more quickly to changes in C isotopes than does the SOM as a whole (Ladd *et*

al. 1992, Bosatta and Ågren 1994). Bosatta and Ågren (1994) found that the relationship between biomass C and total soil C could be described by the microbial growth rate (μ) and microbial mortality. Because μ is controlled by the quality and availability of substrate, they proposed that C quality (q) is the primary factor. Substrates that have high q values have a higher initial rate of decomposition, but result in overall higher stored C values. Such a relationship was observed by Nicolardot *et al.* (1994) when ^{14}C-glucose and ^{14}C-holocellulose (cellulose + hemicellulose) were added to soil. Both substrates were mineralized to the same degree, however more glucose-derived ^{14}C than holocellulose-^{14}C remained in the biomass.

In a study examining the transformation of ^{14}C from glucose and from ryegrass into soil biomass (Wu *et al.* 1993), moderate rates of substrate addition initially raised the total biomass C content. However, at the end of the incubation (100 d for glucose-amended soils, 145 d for ryegrass-amended soils), the total biomass-C in the soil was no greater than at the beginning of the experiment, but the biomass now contained ^{14}C. Also, the total quantity of C now in the biomass was the same as that for the non-amended, incubated control. The biomass is a very dynamic soil C fraction, but it also has steady-state attributes.

The biomass in a soil previously amended with sludge was monitored for 87 wk following the last addition of sludge (Boyle and Paul 1989). The biomass decrease was first order for the first 50 wk ($k = -0.06$ wk^{-1}) as the biomass responded to the consumption of the additional substrate. The researchers calculated a biomass turnover time for the first year of the study was 16.7 wk. I think that this value should be used cautiously because a true turnover time should be obtained from a system at steady-state; the conditions described for this experiment indicate that this was not the case.

The humic materials are an interesting component of SOM, particularly when considered in contaminated soil systems. The classical definition of humic substances is: "A category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in colour, of high molecular weight, and refractory" (Aiken *et al.* 1985). The classical components of humic substances are defined operationally as humic acid, fulvic acid, and humin.

Humic acid: "The fraction of humic substances that is not soluble in water under acidic conditions (pH<2), but is soluble at higher pH values."

Fulvic acid: "The fraction of humic substances that is soluble in water under all pH conditions."

Humin: "The fraction of humic substances that is not soluble in water at any pH value." (all from Aiken *et al.* 1985)

The study of humic acids is ongoing, with a rich historical literature. As early as 1910, firm statements as to the complexity and heterogeneity of humic substances were published (Schreiner and Shorey 1910). It is generally accepted, however, that humic acids are high molecular weight, polymeric structures that have a high phenolic content (Andreux *et al.* 1990). Elucidation of the structure of humic substances is evolving slowly as new tools of chemical analysis arise. Primarily through the application of data collected from pyrolysis-field ionization mass spectrometry in a novel computer program designed to calculate 3-dimensional chemical structures, a structural model of SOM has been proposed (Schulten and Schnitzer 1997). This model is based on a molecular weight of

5547 g mol^{-1} for humic acids, and an elemental composition of 66.69% C, 6.09% H, 25.96% O, and 1.26% N. The conformation of the proposed model is driven by the presence of phenolic-OH groups, and -COOH groups, which control the exchange capacity of the macromolecule; and by the presence of hydrophobic aliphatic hydrocarbons, fatty acid, and fatty acid esters which form bridges of various lengths between the phenolic and other aromatic moieties of the molecule (Schulten and Schnitzer 1997). The resulting predicted structure contains voids that are large enough to occlude large molecules such as trisaccharides and polypeptides. The ability of SOM to sorb such macromolecules is well known, and the ability of the model to reflect this contributes to the validation of the model.

Many of the key functional groups of SOM arise from the degradation of lignin, the release of cellular materials from dying plants, and the products of microbial biosynthesis (Andreux *et al.* 1990). Under oxidizing conditions, a "ubiquitous set of reactions" causes phenols to polymerize with themselves, or with peptides, saccharides, fatty acids, into brown, humic-like substances (Andreux *et al.* 1990, Stevenson 1985). Andreux *et al.* (1990) cited studies in which catechol can polymerize under oxidizing conditions with amino acids in the presence of reduced iron or manganese to form high molecular weight polycondensates which are similar in structure and chemistry to terrestrial humic acids. Those polymerized without the metals were found to be similar to fulvic acids. Both polymers contained high quantities of aliphatic and aryl substituent groups. Other reactions which have been proposed to produce humic substances or precursors are β-oxidation, decarboxylation, demethoxylation, all general reactions that occur as part of the biodegradation of organic chemicals.

Rüttiman-Johnson and Lamar (1997) recognized the relationship between the pathways of contaminant degradation and those of humic substances synthesis, and proposed the use of white rot fungi for modifying the phenolic nucleus of pentachlorophenol (PCP) for binding to humic materials. Over an incubation period of 65 d, four different species of fungi (*Pleurotis ostreatus*, *Irpex lacteus*, *Trametes versicolor*, and *Bjerkanda adusta*) were observed to facilitate binding of ^{14}C-PCP in soil cultures. The binding was more pronounced to the humic acid fraction compared to the fulvic acid fraction. Oxidative enzymes (laccases and peroxidases) likely mediated the binding, however the stability of the bound residues was left in doubt. The incubation period was only 65 d, and did not consider the possible release of components from humic materials as part of the dynamic. To elucidate the mechanism of binding, sorption isotherms were derived for anthracene, carbazole, and dibenzothiophene to humic-coated minerals, after establishing that no sorption occurred when the mineral was not coated (Murphy *et al.* 1991). The sorption isotherms were linear, suggesting that the mechanism was partitioning.

Another study, proposing enhanced humification as a bioremediation strategy, incubated 2,4,6-trinitrotoluene (TNT) with humic materials contained within a dialysis bag (Held *et al.* 1997). In the absence of a TNT-adapted microbial culture, no sorption of TNT to the humic materials was observed. However, when the culture was included, the disappearance of the TNT was complete within 48 h for a range of quantities of humic materials. They found that the second metabolite of TNT metabolism, 2,4-

diaminotoluene, was microbially transformed into the humic materials, likely via a generic peroxidase enzyme.

Delta ^{13}C values have been used to describe the relative turnover rates of organic materials in soils and the relative rates may be: humic acids > bulk organic C > humin (Lichtfouse *et al.* 1995). It has also been suggested that humin may contain up to 50% of the organic C in the soil (Vandenbrouke *et al.* 1985). Since humin is a persistent and substantial soil C fraction, it cannot be ignored as a potential sink for xenobiotic C.

A technique has been proposed recently for fractionating humin. The methylisobutylketone separation procedure distinguishes four components of humin: bitumen, bound humic acids, bound lipids, and insoluble residues (Rice and MacCarthy 1992). The bitumen is the largest component, but the bound humic acids seem to be the most reactive (Rice and MacCarthy 1992, Malekani *et al.* 1997). The surface of the humin has been characterized using N_2 adsorption and small-angle X-ray scattering, and it is very porous. Of the components, the bound humic acids seem to have the greatest degree of surface porosity and, therefore, they have the highest surface area. Presumably, this surface area is important in the reactivity of the bound humic acids.

Xie *et al.* (1997) found that organic chemicals could be sorbed by the humin fraction. They sought to determine whether the humin components favoured different chemical types. The more hydrophobic pesticides examined were preferentially associated with the bound lipid fraction, and that the ^{14}C label on pesticides such as 2,4-D was primarily detected in different fractions when it was ring-labeled compared to side-chain labeled, likely due to the variation in the chemical nature of the sorption sites and the part of the pesticide involved in the reaction. The binding of chemicals to whole soil was not correlated with their binding to humin and its fractions.

The foundation for modeling C transformations in soils is derived primarily from the literature dealing with degradation of plant material. Plant tissues are complex and diverse substrates for soil microorganisms. The transformations of plant-C in soil may be similar to those undergone by contaminant-C. As a starting point for this thesis, consider the model for plant tissue transformations proposed by Sallih and Pansu (1993; Fig. 1-3).

This simple model divides the input material, plant tissues, into labile and resistant components. These components each have unique kinetic coefficients describing the rate at which they enter the soil organic C system. The components enter the system, and from there a multitude of transformations and cycles are possible. Ultimately, oxidation to CO_2 results in the plant-derived C leaving the system, however provisions are made to describe a series of potential cycles through a labile OM pool and a stable OM pool. The labile pool would include microbial biomass, possibly water soluble C, and other readily cycled C. The stable pool is made up of stable humified OM (perhaps including humin), part of which may be chemically stabilized OM.

Key to this model is the division of the plant materials into labile and resistant components. A parallel may be drawn with contaminant-derived C in that crude oil materials are commonly divided into "degradable" (*n*-alkanes, isoprenoids, mono-, di-, and tri-cyclic aromatics) and "slowly degradable" (asphaltenes, NSOs) fractions (McGill *et al.* 1981). From this starting point, it is reasonable that much of the transformation and cycling described by Sallih and Pansu (1993) will be similar to that undergone by contaminant-C. The major goal of bioremediation has appeared to bypass the potential of

the SOM components, focusing instead on the mineralization of the contaminant directly to CO_2. In an effort to fill this gap, this thesis shall endeavour to examine the SOM transformations.

Metabolism of exotic C

The term "exotic C" is used in this thesis to refer to carbonaceous substrates that are applied to the soil and not obtained naturally from within the soil. The fate of a variety of C substrates in soil has been examined for a variety of reasons. Many examples were discussed here for the information their metabolism in soil provides about the dynamics of soil C fractions. The most common exotic substrates applied to soil are glucose (Guzev *et al.* 1997, Ladd *et al.* 1992, Sato and Lee 1996) and cereal straws (Cogle *et al.* 1989, Sallih and Pansu 1993, Summerell and Burgess 1989, Voroney *et al.* 1989). These studies have provided the groundwork for studying the less easily described organic wastes and contaminant mixtures.

Varying the source of added C is a useful tool for examining C dynamics in soil. For instance, the application of glucose to soils has been noted to have a "priming effect" on the mineralization of SOM, *ie*, over a sufficient period of time, the difference between the amount of CO_2 evolved from a glucose-amended soil and a non-amended control may be greater than the amount of glucose added. This priming effect can also occur on the starch and cellulose polymers present in some modern plastics, and therefore, may cause an overestimation of their biodegradability (Shen and Bartha 1997). Also, it has been observed that the mineralization of certain complex C substrates may be enhanced by the presence of other, more readily mineralizable C substrates, as is the case with the enhanced degradation of lignin in soil systems that include cellulose (Entry and Backman 1995).

Ågren and Bosatta (1987) recommend that the degradation of freshly added organic substrates to soils be seen as "a continuum beginning with the input of fresh organic materials and leading to the formation of refractory humic substances." This continuum has been well described for several exotic C sources and reviewed in this introduction. The next direction in this field is to further examine this continuum as it occurs in contaminated soils.

Studying C in Soils

In researching the literature for this project, the vast number of techniques that have been employed to study C in soils became apparent. Researchers have used synthetic soils consisting of the soil components being studied in a sand matrix (Murphy *et al.* 1990). They have applied contaminant compounds to clean soils (Kanaly *et al.* 1997, Guzev *et al.* 1997, Hatzinger and Alexander 1995). Researchers have examined components of contaminated soils (Pavlostathis and Mathavan 1992), and they have spiked known compounds into field-contaminated soils (Deschênes *et al.* 1996). Obviously, the examination of individual compounds in field contaminated soils is the best way to study contaminated soils, however it is often difficult to identify specific compounds to seek. The use of pure or artificial systems is important, as it allows specific interactions to be identified without the interference of the rest of the soil components and interactions. The application of radiolabeled (or otherwise identifiable) compounds to

field-contaminated soils has the advantage of allowing the researcher to use a realistic model system and still be able to trace elements of an individual compound. There are still problems in that the freshly added compound is not likely to behave exactly as it would if it had weathered in the soil with the rest of the contaminants, but the findings are still useful if this caveat is not forgotten.

In an attempt to clarify some of these issues, ^{14}C-labeled phenanthrene was applied to 16 soils and allowed to age for 200 d. In 15 of these soils, the percent phenanthrene mineralized decreased significantly from its fresh application (time 0) to the end of the incubation (Chung and Alexander 1998). A similar decrease in the extractability and biodegradability was observed for phenanthrene and 4-nitrophenol aged in a loam, a muck, and an aquifer sand (Hatzinger and Alexander 1995. It is then reasonable that a "spike" of chemical, freshly added to a weathered field-contaminated soil in the lab, will behave differently from its counterpart present in the contamination.

Research

This research addressed the concerns of enhancing bioremediation of soils by clearly examining the fate of xenobiotic C in weathered contaminated soils in model soil systems maintained with a minimum of intrusion. The impetus for this research project began as a part of the University of Alberta research initiative sponsored by the Environmental Science and Technology Alliance of Canada (ESTAC). The primary focus of this project was to understand and enhance the desorption of contaminants from soils as a means of enhancing their potential for remediation. The first experiment I conducted, described in Chapter 3, was to assess the potential of these soils for passive bioremediation strategies. I monitored the respiration rates of the four core "ESTAC" soils for 10 wk, and measured the change in DEO after the incubation period. In three of the four soils the amount of C evolved as CO_2 was much smaller than the amount of C lost from the DEO fraction. This lead to the formulation of my research theme: *to account for and describe the transformations of contaminant-derived C within the SOM of weathered contaminated soils.*

Two of the four ESTAC soils were selected for study in this thesis, based on contamination type (one was oil-contaminated, the other creosote-contaminated), and on their supply and availability (the oil-contaminated soil was collected near Devon, Alberta and the creosote-contaminated soil was collected from a site in the city of Edmonton). While the ESTAC stocks of each soil were used for the experiment described in Chapter 3, new samples of the two selected soils were obtained. These samples were used in the experiments described in chapters 2, 4, 5, and 6, and they have a lower DEO content than the ESTAC stocks. Model organic contaminants (MOCs) were chosen for each soil, based on the type of contamination present. Carbon-14 labeling of these compounds simplified tracking the fate of this C in soil. The straight chain, C_{18} alkane, octadecane, was selected for the oil-contaminated soil, and the 4-ring PAH pyrene was selected for the creosote-contaminated soil. I chose to use MOC mineralization to CO_2 and the traditional soil C fractions water soluble-C, biomass-C, humic-C, and DEO-C as possible fates (Fig. 1-4), based on a C transformation model in which MOC-C initially in the DEO-C must pass through the biomass-C to be transformed into water soluble-C, and humic-C. The proposed C fractionation scheme is shown in Fig. 1-5.

A model designed by Sallih and Pansu (1993) was presented previously (Fig. 1-3). This thesis will endeavour to modify the model described in Fig. 1-3 for application to contaminant-C. There are some problems with this model: the unexplained circle in the middle of the diagram, through which all reaction seem to pass; the production of CO_2 does not come directly from the biomass; no indication of reaction rates are given. These points shall be clarified.

This challenge of describing the fate of MOC-C is first confronted in Chapter 4, which describes the fate of model organic contaminants during a 12-month incubation of the two soils. The results of the first 12 wk of this experiment inspired the incubations described in Chapters 5 and 6. Chapter 5 reports on the interactions between MOCs, contaminant NAPLs, and their location in the soil. Chapter 6 reports on the fate of simpler exotic C substrates in these soils as well as on the behaviour of the MOCs when cross-contaminated into the non-type soil.

The experiment described in chapter 2 does not conform to the incubation format of the experiments in chapters 3, 4, 5,and 6. The experiment in chapter 2 examined the potential enhancement of bioremediation in vegetated versus non-vegetated systems. Due to a combination of a lack of encouraging results for this experiment and the encouraging early findings of the first radiolabeled-MOC incubation (chapter 4), this line of research was not pursued further.

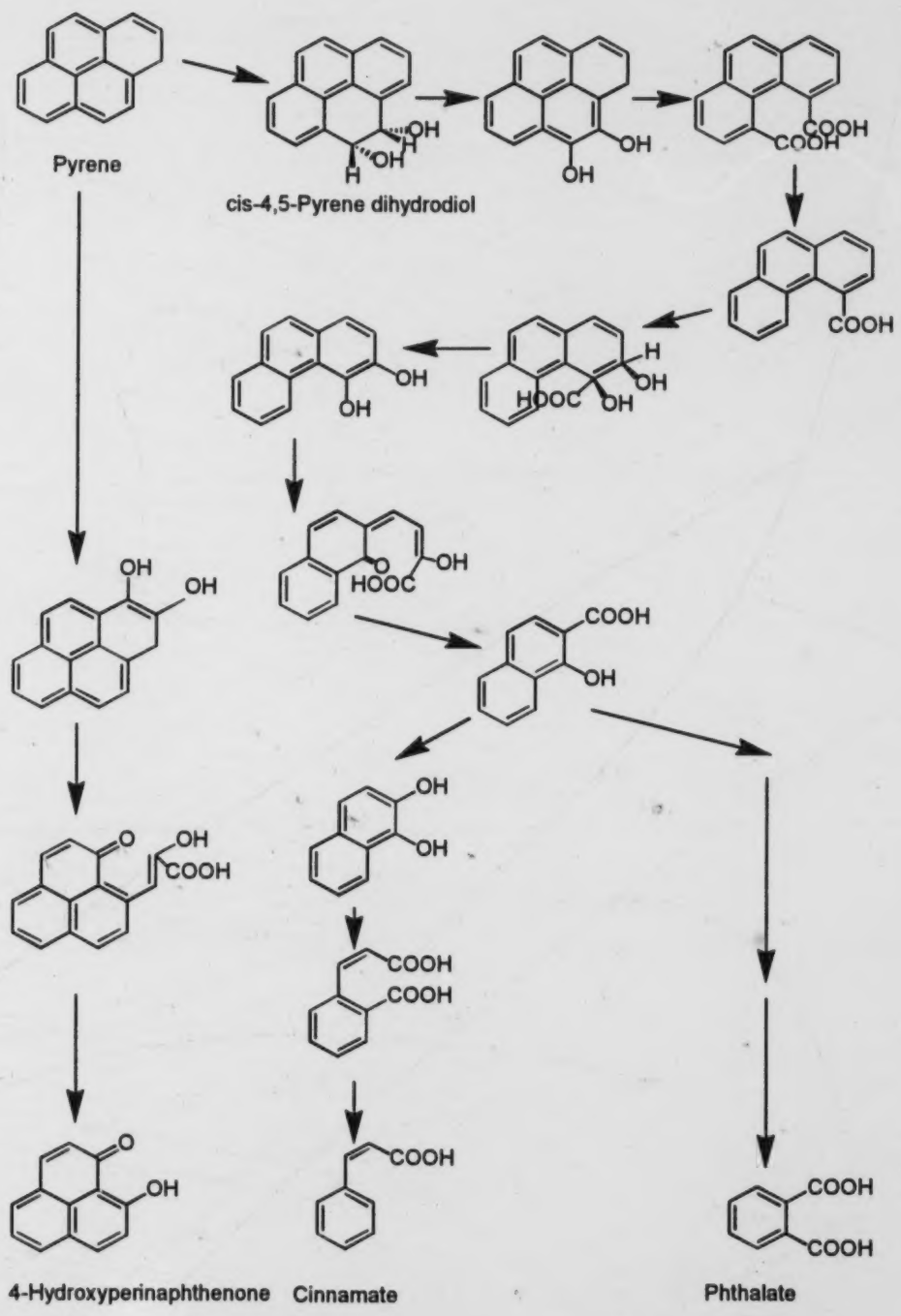

Figure 1-1. Three proposed pathways for the metabolism of pyrene by *Mycobacterium* sp. PYR-1 (from Cerniglia 1992).

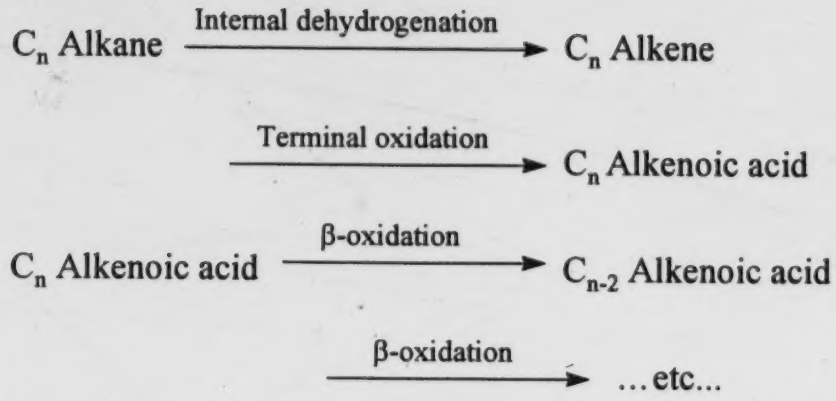

Figure 1-2. The pathway for β-oxidation of alkanes in soils (from Parlanti *et al.* 1994).

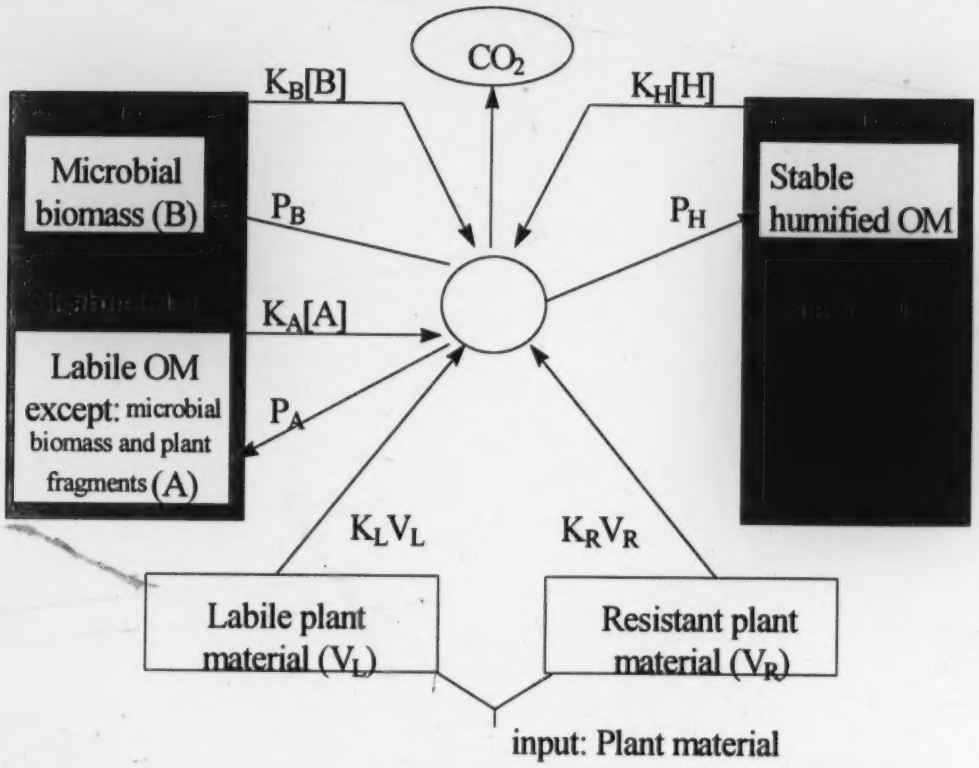

Figure 1-3. A proposed model describing the fate of plant-derived C decaying in soil (Sallih and Pansu 1993). K_L, K_R = kinetic coefficients of decay in compartments V_L and V_R; P_A, P_B,P_H = input proportions into A, B, H.

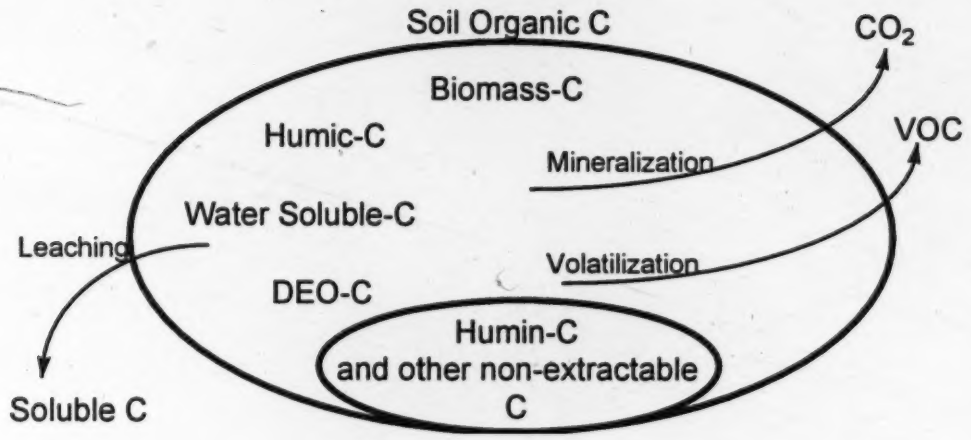

Figure 1-4. A conceptual diagram of soil organic carbon for a contaminated soil showing potential losses from the system and the specific fractions within the total.

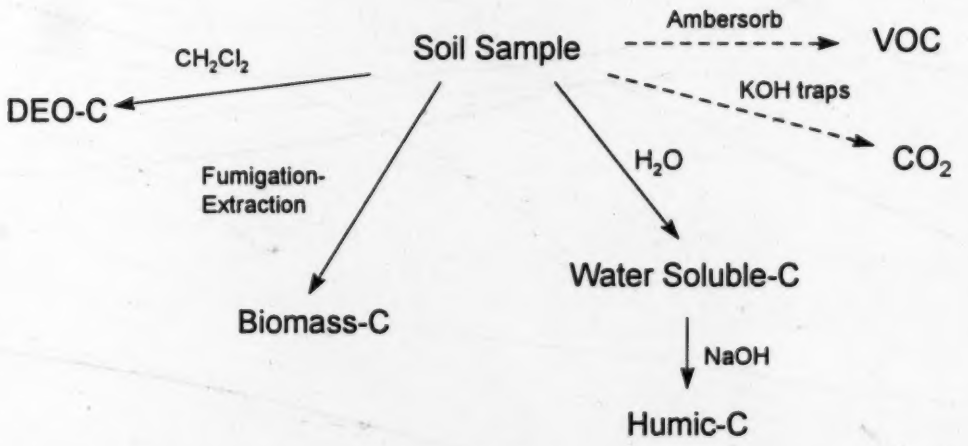

Figure 1-5. The analysis scheme for fractionating soil C. Dashed lines indicate traps, solid lines indicate soil analyses.

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CHAPTER 2

THE POTENTIAL FOR PLANT-ASSISTED BIOREMEDIATION OF TWO HYDROCARBON-CONTAMINATED SOILS

Introduction

Bioremediation is a popular option for cleaning up soils contaminated with hydrocarbons. The least intrusive systems, such as land-spreading, or low-input systems, such as biopiling, often take advantage of the indigenous microbial communities which may have adapted to the local contamination. These systems may benefit from maximizing the potential of the ecosystem to metabolize the contaminant (Bossert and Compeau 1995).

Phytoremediation, in the most direct sense of the word, refers to the removal of contaminants from soil through plant uptake. Additionally, plants may serve to enhance bioremediation by providing an enriched habitat for soil microorganisms in the rhizosphere, breaking and rebuilding soil aggregates, protecting the soil surface from erosion, and creating channels into the soil for aeration and microbial habitation (Cunningham and Ow 1996; Shimp *et al.* 1993).

Shimp *et al.* (1993) list six reasons for the inclusion of plants in remediation systems: 1) Plants utilize solar energy, thereby providing additional energy to the bioremediation system; 2) Plant development and tissue subsamples may be used as indicators of contaminant toxicity or remediation success; 3) Plants may reduce the spread of a contaminant in soil by removing water from the soil, maintaining a small water potential near the surface; 4) The rhizosphere is densely colonized by a variety of microorganisms which may be able to degrade a wide variety of organic contaminants; 5) Many plants have mechanisms for transporting oxygen to the rhizosphere; and, 6) Plants are aesthetically pleasing. A seventh reason could also be included, 7) Plants physically protect the surface of disturbed soils from the erosive actions of wind and water (Schnoor *et al.* 1995).

The rhizosphere is the zone of C and microbial enrichment that immediately surrounds the plant root (Wild 1988). The root provides surfaces for microbial colonization additional to the soil solids, and the root exudates and exfoliated meristem cells provide rich carbon and energy sources for the microbial community. Up to 100-times more organisms may colonize the root surface than do the bulk soil (Paul and Clark 1989). Different plant species support different communities, however the communities are typically much larger than in the surrounding soil (Shimp *et al* 1993). Even in contaminated soils, significantly more microorganisms (Lee and Banks 1993) or larger microbial biomasses (Walton and Anderson 1990) are found in vegetated soils than in non-vegetated soils.

Plant root exudates are generally simple organic compounds such as organic acids, amino acids, fatty acids, etc. (Hsu and Bartha 1979; Shimp *et al.* 1993) which are readily used as cosubstrates during the metabolism of more complex, less desirable organic contaminants (Hsu and Bartha 1979). Plant root exudates may arise from "leaks" of cellular materials when sloughed root cap cells rupture, "secretions" of extracellular plant

compounds, and "mucilages". The secretions and "leaks" may include enzymes, aliphatic and aromatic hydrocarbons, amino acids, sugars, and low molecular weight carbohydrates (Burken and Schnoor 1996). Mucilages are gel-like materials composed of low molecular weight carbohydrates. The addition of dead root tissue, or of the entire root following plant senescence to soil is termed rhizodeposition. The extent of rhizodeposition and exudation depends on many plant characteristics. The entire rooting system of an annual plant decays in the soil following senescence whereas only a portion of the root system of a perennial is decomposed. To examine the role of plant exudates in facilitating bioremediation, Reilley *et al.* (1996) stimulated non-vegetated soils spiked with ^{14}C-pyrene with applications of organic acids to mimic root exudates. They measured greater quantities of $^{14}CO_2$ evolving from these soils than from spiked, non-vegetated, non-amended soils, providing evidence that the presence of root exudates as cosubstrates may be instrumental in plant-enhanced systems.

Plant roots may also reach great depths, loosening the soil, allowing oxygen diffusion and nutrient delivery to contaminated sites within the soil profile which otherwise may have been "starved". The delivery of oxygen to subsurface soils may be done via diffusion through a soil whose pore space has been increased by root penetration. Ameliorating species could be selected using a comparison of mean penetration depth of plant roots with the depth of contamination (Shimp *et al.* 1993).

The purpose of this experiment was to examine the potential for plant-assisted bioremediation of two hydrocarbon-contaminated soils by comparing changes in dichloromethane extractable organic materials (DEO) in these soils, planted to five plants and compared with non-planted and time = 0 controls. The five plants, *Triticum aestivum* (wheat), *Brassica rapa* (canola), *Helianthus annuus* (sunflower), *Vicia faba* (fababean), and *Trifolium hybridum* (alsike clover), were chosen to represent a range of commercially and agriculturally important crop species.

Materials and Methods

Soils

Samples from two weathered contaminated soils were used in this experiment: an oil-contaminated soil and a creosote-contaminated soil. The oil contaminated soil, collected near Devon, Alberta is loam textured, pH 7.7 and contained 5.6 mg DEO g^{-1} soil from an oil-well blowout in 1947. The creosote contaminated soil, collected from a site in Edmonton, Alberta is silty clay textured, pH 7.3, and is contaminated with 1.5 mg DEO g^{-1} soil, residual from a wood treatment plant on the site between 1924 and 1988. A third "soil", a pristine (non-contaminated) potting mix that was a blend of peat, sand and vermiculite, was included as a soil control.

Plants

Five plant species, common in prairie cropping systems were selected: *Triticum aestivum*, cv. Neepawa (wheat), *Brassica rapa*, cv. El Dorado (canola), *Helianthus annuus*, cv. DO827 (sunflower), *Vicia faba*, cv. Orion (fababean), and *Trifolium hybridum*, cv. Dawn (alsike clover).

Experimental

The experimental design was factorial: 3 soils × 5 plant species. A subtreatment was applied to the legumes (clover and fababeans): fertilization with P and K, no N. Each species was seeded to a depth of ~1.5 times the seed diameter into each of the three soils in triplicate. *T. aestivum*, *B. rapa*, *V. faba*, and *T. hybridum* were planted in 15-cm (diameter) pots containing ~2 kg soil, and the *H. annuus* was planted into 17.5-cm pots containing ~2.25 kg soil. These pots were placed on top of inverted 10-cm pots within 15-cm MacConkey pots (Fig. 2-1). The environmental conditions were set at 21°C for 16 h with a light intensity of 275 μE (day period) and 18°C for 8 h (night period). The humidity was approximately 25% R.H. All pots were watered daily. *T. aestivum*, *B. rapa*, *V. faba*, and *H. annuus* were harvested after 3 months of growth. *T. hybridum*, a two season crop, was harvested after 6 months. As incubation controls, unplanted pots of the experimental soils were incubated with the planted pots. Each pot received fertilization monthly with a solution of 20-20-20 (N-P_2O_5-K_2O) equivalent to 0.0125 g N pot^{-1}. The unplanted, *V. faba*, and *T. hybridum* pots were further replicated to include a 0-20-20 treatment, receiving the same quantity of P and K as did the +N pots. When harvested, the above-ground plant materials were collected in brown paper bags and dried at 35°C to a constant weight. The soils were collected in plastic bags and stored at 4°C for further analysis.

Leachate

At the end of each month, leachate subsamples were collected immediately after watering the pots so that the water ran through the soil and filled the MacConkey pot (Fig. 2-1). These samples were analyzed for dissolved organic carbon (DOC) on an Astro 2001 Series 2 Soluble C Analyzer with an ultraviolet source. The analysis used 1 M sodium persulfate as the oxidizing agent, and 85% phosphoric acid as the sparging solution. The calibration standard was a 100 mg L^{-1} aqueous solution of potassium acid phthalate.

Soil and Plant Analysis

The DEO were Soxhlet-extracted from the soils with dichloromethane. Subsamples of ~10 g (oven dry basis) soil were placed in cellulose thimbles and mixed with an equal amount of anhydrous $MgSO_4$ to ensure a dry extraction. Dichloromethane (200 mL) was cycled through the Soxhlet apparatus overnight at a rate of 4 cycles h^{-1}. The heat source was a sandbath set at 75°C. The collected extract was reduced under vacuum to < 5-mL. This extract was quantitatively transferred to a 25-mL volumetric flask and brought to volume with fresh dichloromethane. A 5-mL aliquot was transferred to a pre-weighed aluminum dish and the dichloromethane allowed to volatilize. The tarry residue remaining was weighed on a precision balance (to 4 decimal places) and designated total DEO.

The above-ground plant tissues were ground in a Wylie mill to pass through a 0.5 mm mesh screen. Ground plant tissue subsamples (1-3 g) were weighed into cellulose thimbles and Soxhlet extracted overnight with 200 mL dichloromethane. Plugs of glass wool held the ground plant material in the cellulose thimbles to prevent its floating during extraction. Following Soxhlet extraction, the plant extracts were reduced under vacuum

to < 5 mL and quantitatively transferred to a 10 mL volumetric flask and brought to volume with fresh dichloromethane.

Both soil and plant extracts were stored in borosilicate glass scintillation vials and immediately weighed before storage at 4°C.

Selected plant extracts (*V. faba* and *B. rapa* from each of the three soils) were analyzed for evidence of contaminant-hydrocarbon uptake using a Carlo Erba 5160 HRGC connected to a Finnigan 4500 MS. Prior to analysis the extract residues were re-dissolved in hexane and passed through a column of anhydrous aluminum oxide in order to collect specifically the aromatic and saturated fractions. The HRGC had a Restik XTI-5 fused silica column (30 m, 0.25 mm ID, 0.25 μm df.) and the carrier gas was helium (35 cm s^{-1}). The GC/MS used multiple ion detection (0.053 s $mass^{-1}$). The ion source temperature was 150°C, the electron multiplier was 1350 ev, and the transfer line temperature was 300°C. The optimal run time for the fababean tissues was 40 min and for the canola tissues was 20 min.

Statistics

A one-way ANOVA, with an LSD means separation was conducted using SAS (Windows 6.10) PROC GLM to determine if there were differences among treatments, within soils. The same test was conducted on the above-ground dry matter measurements to determine if there were differences in plant yields among plants, within soils. Significant differences in the quantity of C measured in the collected leachates among months, within plant-soil treatments were analyzed as a "split plot in time" with means separation analysis.

Results

The yield of above-ground dry matter from plants grown in the contaminated soils was the same as, or less than the yield of dry matter from the same plant grown in the control soil, except for one treatment (Fig. 2-2). The fababeans grown in the creosote-contaminated (-N) soil had a larger above-ground dry matter yield than did the fababeans grown in either the control soil or the oil-contaminated soil. Unfortunately, none of the replicates for the fababeans in creosote-contaminated (+N) soil succeeded in establishing themselves, likely due to poor soil physical conditions rather than chemical inhibition.

The monthly leachate data followed the same overall trend for the two contaminated soils (Fig. 2-3). As time progressed, the concentration of C in the leachate generally decreased. The exceptions to this were the sunflower- and fababean- planted oil-contaminated soil in which the C concentration did not change over the 3-month growing period and the fababean- and sunflower-planted creosote-contaminated soil in which the concentration of C in the leachate increased over the 3-month period.

No significant loss of DEO was measured in either contaminated soil during the 3-month growing period (Fig. 2-4). Over the 6-month growing period, a significant loss in DEO was measured in both clover and clover (-N) for both soils (Fig.2-5). These losses were not significantly different from each other, nor were they different from the losses measured in both of the non-planted pots for both soils. Observations made following solvent extraction of both soils and all the plants was the wide range of colours of the extracts of the same soil (yellow to dark brown), and the wide variety of

shades of green for the same plant species, grown in different soils. This prompted further analysis of selected extracts.

Gas chromatography/MS of fababean and canola tissues grown in all three soils, suggests that there may have been uptake of hydrocarbons into the tissues of the plants grown in the oil-contaminated soil (Figs. 2-6; 2-7). This was determined by comparing the scans of the plant extracts from the contaminated soils with those of the plants grown in the pristine control soil. The chromatogram of the plant tissue grown in the creosote-contaminated soil does not differ from the chromatogram of the same plant tissue grown in the control soil. However, the chromatogram of the fababean plant tissue grown in the oil-contaminated soil shows enrichment of C_{16}, C_{17}, and C_{18} hydrocarbons, as well as the hump between 16 and 21 min that is characteristic of naphthenic hydrocarbons. The chromatogram of the canola tissues grown in oil-contaminated soil (Fig. 2-7) shows a hump of unresolved hydrocarbons, likely alkanes, between 10 and 15 min that is not seen in the canola grown in the other two soils.

Discussion

All plants grew poorly on the contaminated soils, in contrast to studies where low levels of contaminated municipal sludge (Baghdady and Saad 1992; Wild and Jones 1992) and PAHs have stimulated plant growth (Sims and Overcash 1983) in a range of plants (*Daucus carota*, *V. faba*, *Secale cerealis*). Decreased plant yields in fuel-contaminated soils have been measured before and are not surprising (Chaîneau *et al.* 1997). These are likely due to poor chemical and physical conditions in the contaminated soils as well as toxicity of high levels of contaminant to the plants.

When the overlying soil was teased away, the fababeans in the creosote-contaminated soil had germinated, but failed to take root. This was likely due to the clodding of the creosote-contaminated soil whenever it was watered, reducing contact between the soil, moisture, and the large seed. It seems that the creosote-contaminated soil in the (-N) pots clumped around the seeds, maintaining the required contact for establishment. This could be a random event, or, more likely, is an artifact of where they were located in the growth chamber relative to the impact of watering, as the (-N) pots were further away from the hose than were the (+N) pots. The oil-contaminated and control soils showed no tendency to clump, rather they "settled" following watering. The seeds of all the other plants included were much smaller than those of the fababean, and likely established in niches around and under the clods in the creosote-contaminated soil.

The loss of soluble C from the contaminated soils was greatest early in the experiment. It may be that the simpler C substrates were metabolized to more soluble forms by soil microorganisms following the first addition of nutrients to stimulate them. Oddly, the sunflower-planted creosote-contaminated soil lost greater amounts of soluble C as the experiment progressed, while the sunflower-planted oil-contaminated soil decreased in month 2, but rose again in the last month. It may be that the organisms stimulated by sunflowers are better able to mineralize contaminants to soluble forms than the organisms favored by the other plants. It may also be that sunflowers produce larger quantities of root exudates and sloughed materials under the conditions of this experiment than do the other plants included.

Based on the DEO data (Fig. 2-3 and 2-4), the presence of plants did remove more contaminants from soil. The only factor in this experiment that did relate to hydrocarbon removal from these soils was time. All of the soils, vegetated and non-vegetated, when incubated for 6 months lost the same amount of DEO. These findings are similar to those of Wiltse *et al.* (1998), who found the no difference in crude oil-derived DEO in soils planted with alfalfa compared with non-vegetated soils after 6 months, but did measure the same significant decrease in the soils compared to their original DEO contents. We can contrast these findings with a number of other experiments that did find enhanced removal of contaminants in the presence of plants. Papers that describe enhanced removal of contaminants in the presence of plants generally tend to look at one specific component of contamination or at "spikes". Wild and Jones (1992) measured significant decreases in the total concentration of 14 PAHs, ranging between naphthalene and coronene in soils after 82 d of carrot growth. Aprill and Sims (1990) measured significant losses of PAHs from contaminated soils with prairie grass vegetation, compared with non-vegetated controls. Reilley *et al.* (1996) measured significantly greater removal of added pyrene and anthracene in petro!eum-contaminated soils planted with grasses or alfalfa compared with non-vegetated controls. Plant-microbe associations known to enhance 2-chlorobenzoic acid disappearance from soils are not as efficient when the contaminant is present in a mixture of contaminants (Siciliano and Germida 1998).

It is possible that our experiment would have benefited from analyzing the DEO extracts for specific contaminants. The experiments cited in which specific PAHs were studied report no data on dichloromethane extractable organic material changes. The sensitivity of analyses for individual components is greater than the sensitivity of the DEO analysis.

Another explanation for the lack of plant-enhanced disappearance of DEO is that both soils included in this studied are *weathered contaminated soils*. Soils that are contaminated with residual heavy materials that remain after the contaminant has aged in the soil, rather than with the individual compounds, light phase contaminants, or pesticides evaluated by others which have dominated this body of literature. The weathering of contaminants in soil may result in the uneven distribution of the contaminant through the soil matrix (Hatzinger and Alexander 1995). Lowest concentrations of contaminant may be located at the surface of aggregates, where microorganisms, water, and air may interact to metabolize the substrate. Highest concentrations may be located within aggregates, where the contaminant is protected from metabolism. Not only may the proportion of the soil explored and modified by the root systems be too small, the part of the soil explored may have too little contamination for the rhizosphere to be of significance to the whole soil.

Several papers have been published which examine plant uptake of contaminants. In agreement with the findings presented here, Goodin and Webber (1995) observed no uptake of anthracene or benzo(a)pyrene by rye, soybean, or cabbage grown in spiked, sludge-treated soil, based on acetone-hexane Soxhlet extracts of the tissues. Methanol extracts of a fescue, sudangrass, and alfalfa, grown in pyrene- and anthracene-contaminated soil also all failed to show evidence of incorporation of these contaminants into plant tissues (Reilly *et al.* 1996). However, plant roots, grown in contaminated soils have been shown to accumulate organic constituents from the contaminant (Schroll and

Scheunert 1992; Wild and Jones 1992). Schnoor *et al.* (1995) categorize contaminant potential for plant uptake by the K_{ow} of the contaminant: direct uptake and accumulation is most likely for compounds with a log K_{ow} between 0.5 and 3; compounds with a log $K_{ow}>3$ are too strongly bound to root surfaces to be transported to above ground tissues and; those with a log $K_{ow}<0.5$ do not sorb sufficiently to be transported through plant membranes.

Our results did suggest, however, that contaminant-derived aliphatic hydrocarbons were recoverable from selected plants (*V. faba*, *B. rapa*) grown in the oil-contaminated soil. Carbon chains of 16, 17, and 18 units were identified in the chromatogram of V. faba that were neither seen in the plant tissues grown in the creosote-contaminated nor the pristine soils. As well, in both the *V. faba* and *B. rapa* unresolved hydrocarbons are observed uniquely in the oil-contaminated soil.

Much of the literature published about plant-enhanced bioremediation of soils has focused on the beneficial aspects. Our examination of DEO contents before and after incubation and in vegetated and non-vegetated controls would suggest that the five plants selected in the two soils studied do not enhance bioremediation. However, the hints of plant accumulation of hydrocarbons from the oil-contaminated soil suggest that plants may be a fate of xenobiotic C in soils.

The results of this paper indicate that the role of plants in bioremediation systems, both as enhancers of bioremediation systems and as possible sinks of contaminant C should be studied further. Gross measurements of DEO materials extracted from cropped soils compared to those from non-cropped controls show no evidence of plant facilitation of bioremediation. However, the GC chromatographs of dichloromethane extracts of fababean and canola tissues indicate that unique hydrocarbons are present in the plants grown in oil-contaminated soil which do not appear in the plants grown in pristine or creosote-contaminated soil. The potential role of plants as a sink for some contaminants should be examined further.

Careful attention must be given to the age of the contaminant in the soil and to a careful accounting of both individual pollutants and the total content of contaminant C. Due to the increasing complexity of hydrocarbon residues as they weather in soil, conclusions drawn from artificially contaminated systems should be regarded as only a starting point for further research, rather than as models for field situations. Furthermore, future experiments into the potential for bioremediation should use both gross analyses (*ie*, DEO, total PAHs) as well as compound-specific analyses. Our work, in comparison with published literature, suggests that one measurement without the other may yield misleading results.

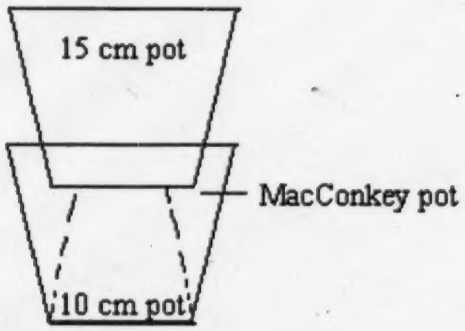

Figure 2-1. Leachate collection apparatus. The plants were planted into the upright pot placed on top of the inverted pot. The leachate was then collected in the MacConkey pot.

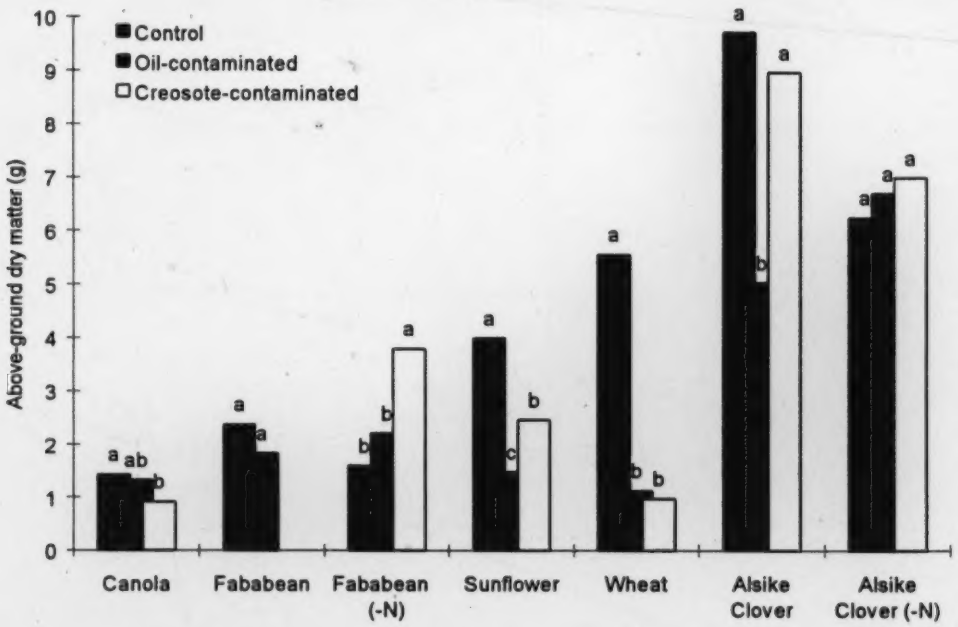

Figure 2-2. Above-ground dry matter yields from five plant species grown in each of two contaminated soils and one pristine control soil. Bars topped by the same letter, within a crop treatment, are not significantly different ($p<0.05$).

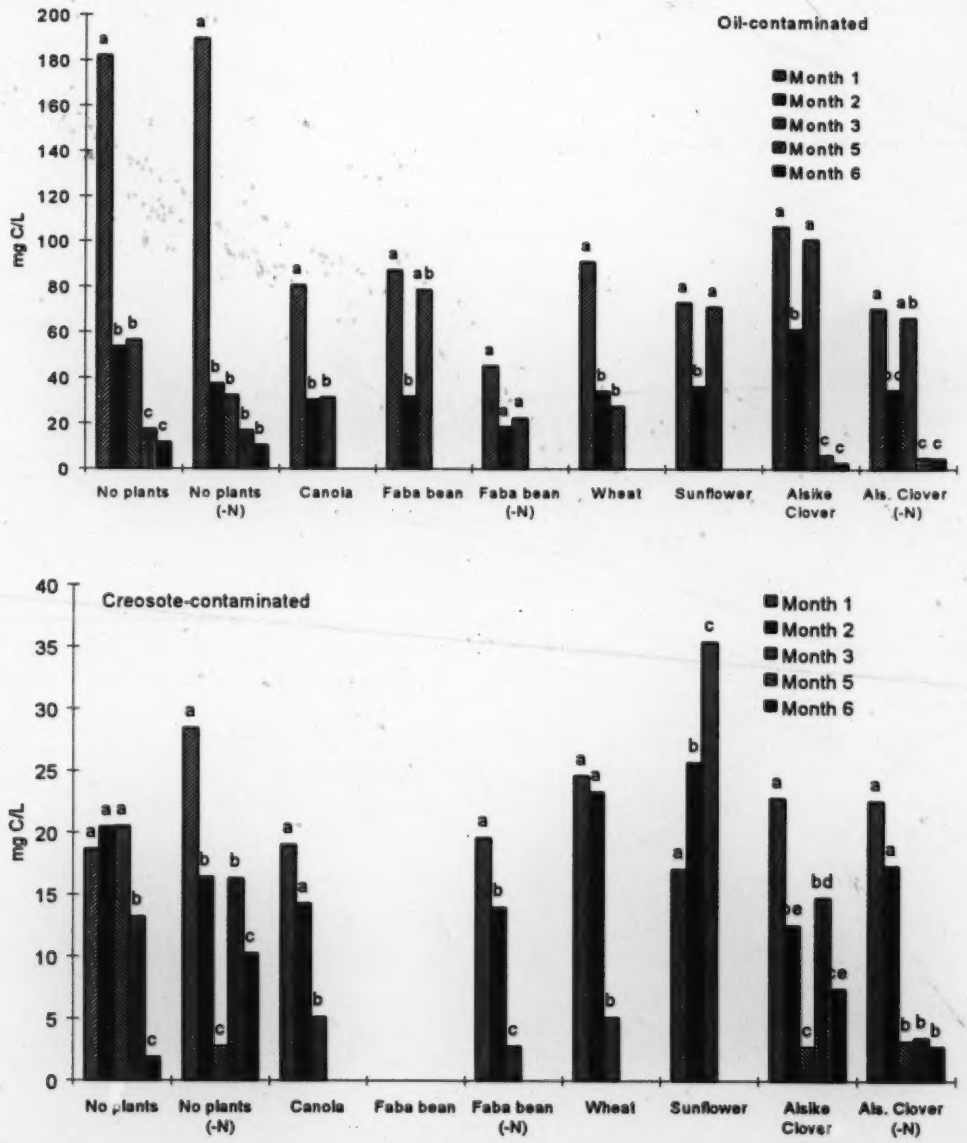

Figure 2-3. Soluble organic C contents of leachate collected from two contaminated soils over 6 months (control and alsike clover) and 3 months (canola, fababean, wheat, and sunflower). Comparisons are only valid within plant ($P<0.05$). (a) Oil-contaminated soil, (b) Creosote-contaminated soil.

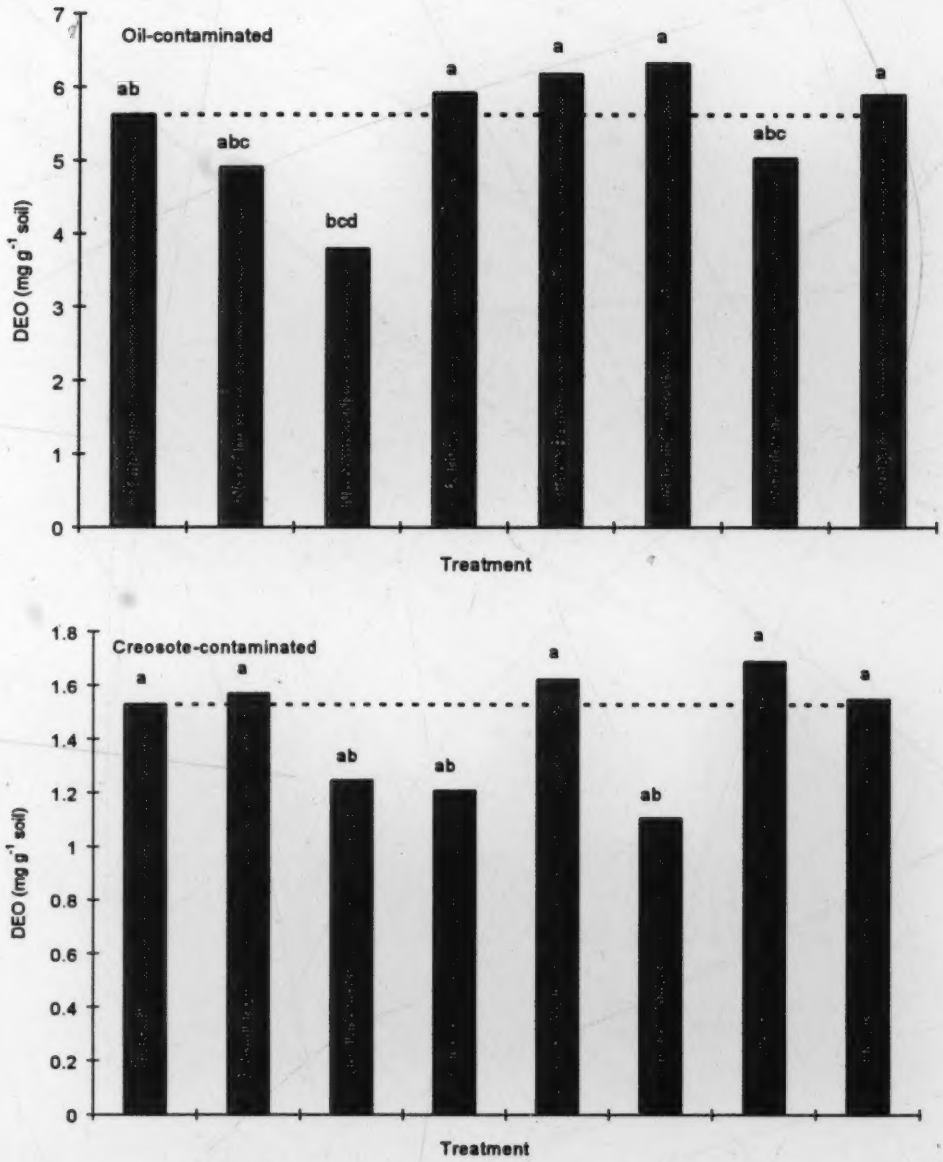

Figure 2-4. Soil DEO concentrations at time=0, and t=3 months (no plants, no plants (-N), canola, fababean, fababean (-N), sunflower, and wheat). Bars topped by the same letter are not significantly different ($p<0.05$).

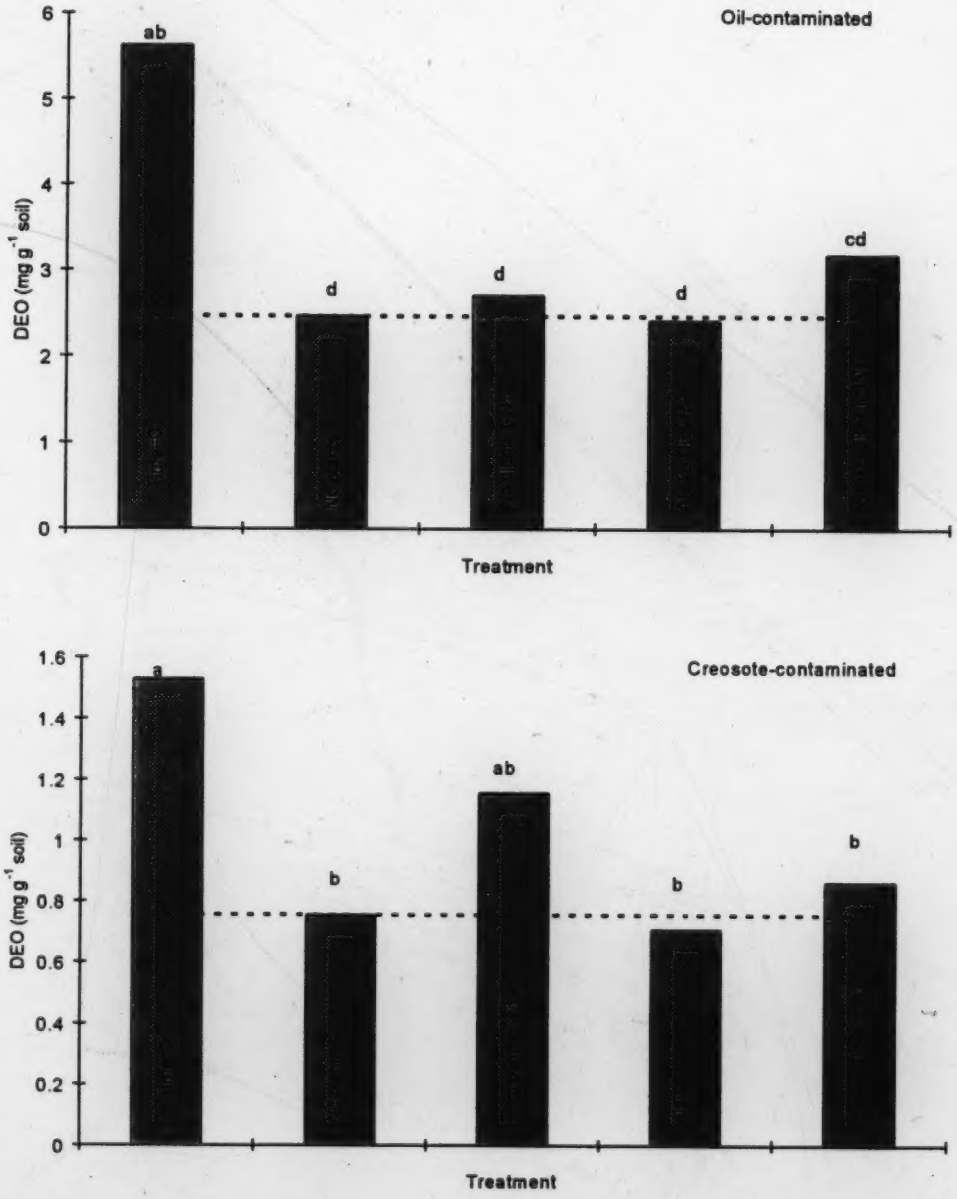

Figure 2-5. Soil DEO levels at time=0, and t=6 months (no plants, no plants (-N), alsike clover, and alsike clover (-N)). Bars topped by the same letter are not significantly different ($p<0.05$).

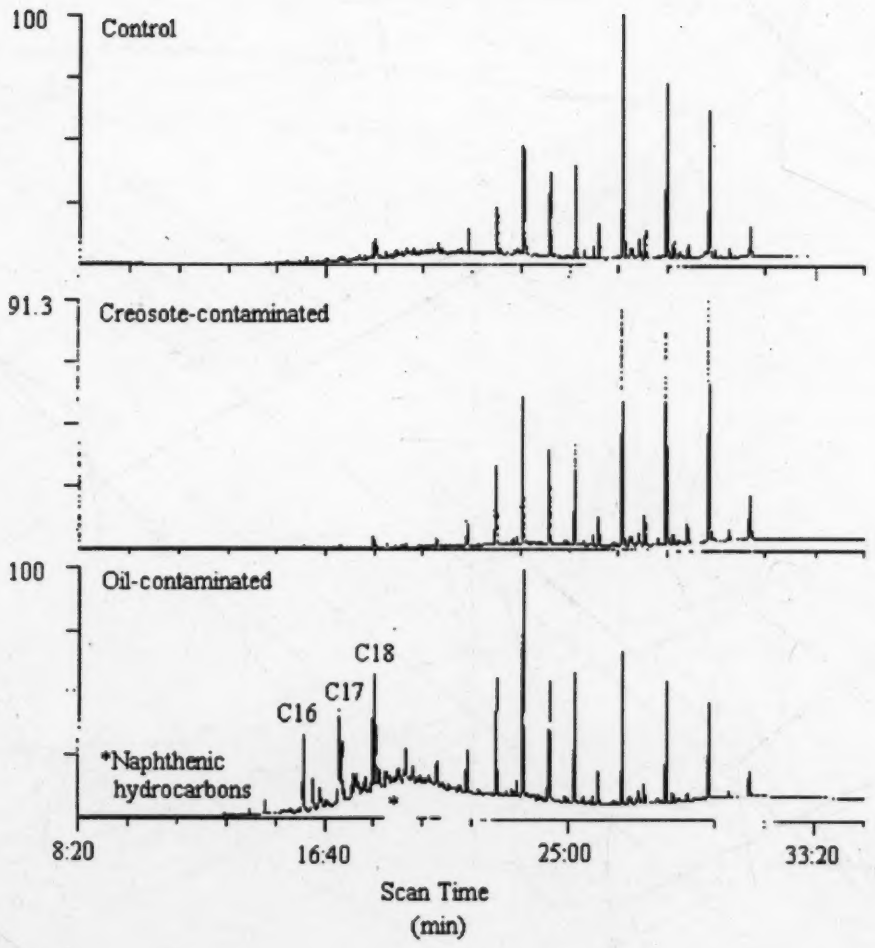

Figure 2-6. GC/MS chromatograms of the tissue extract of fababean (+N) grown in all three soils. Note the non-plant hydrocarbons eluting between 12 min and 21 min in the chromatogram for the oil-contaminated soil.

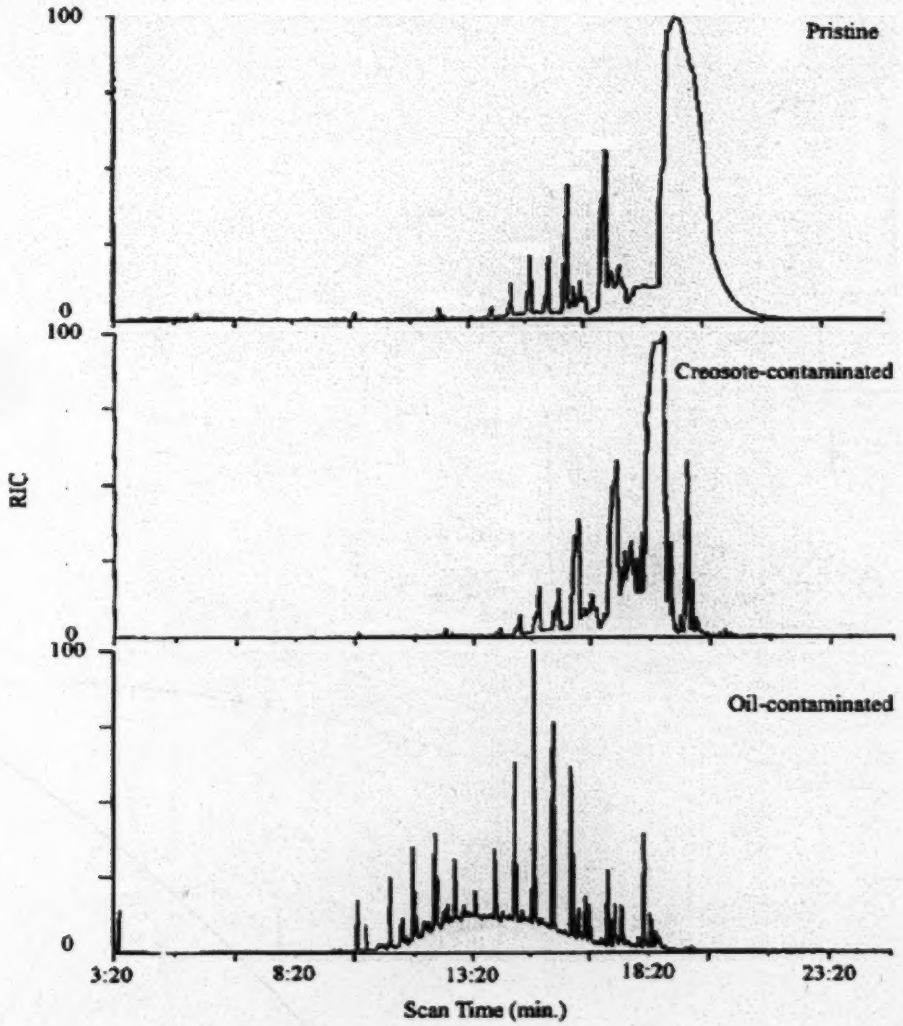

Figure 2-7. GC/MS chromatograms of the tissue extract of canola grown in all three soils. Note the non-plant hydrocarbons eluting between 10 min and 15 min in the chromatogram from the oil-contaminated soil.

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CHAPTER 3

THE POTENTIAL FOR PASSIVE BIOREMEDIATION OF FOUR HYDROCARBON-CONTAMINATED SOILS

Introduction

Soils are often contaminated with hydrocarbon compounds anthropogenically. Such sites may be identified when industrial facilities are decommissioned and the underlying soil found to contain recoverable hydrocarbon residues. Other contaminated sites may arise from more traumatic contamination events, such as oil well blow-outs.

There are two main sources of hydrocarbon contaminants to soils considered here: petroleum and creosote. Petroleum hydrocarbons include aliphatics: both straight and branched chain alkanes, cycloalkanes, and alkenes; aromatics: monoaromatics and polyaromatic hydrocarbons (PAHs); asphaltenes, and resins or NSO-containing compounds. Although the resin fraction is not a pure hydrocarbon it is often included in lists of petroleum hydrocarbon constituents. When present in soils in sufficient quantities, contaminant hydrocarbons disrupt plant growth, pose water quality risks, and impair soil physical conditions, especially water relations (McGill *et al.* 1981). Creosote is characteristically a mixture containing oils, Dense Nonaqueous Phase Liquids (DNAPLs) and pitch. It is produced by the destructive distillation of coal in coke ovens or retorts. Creosote consists of various coal tar distillates (200°C - 400°C) that contain PAHs (~85 % w/w), heterocyclic polyaromatic hydrocarbons (~3 % w/w), and phenols (~12 % w/w) and is used mostly as a wood preservative (Pollard *et al.* 1994). PAHs are compounds made of conjoined aromatic rings and include representatives varying from 2-member rings such a naphthalene, to n-member rings such as pyrene and chrysene (4 rings; Liu *et al* 1992; Song *et al* 1990; Fig. 3-1). These compounds are commonly found in coal tars and creosote (Peters and Luthy 1993). PAHs are of particular concern in the environment because fluoranthene (3 rings), benzo[a]anthracene (4 rings), and benzo[a]pyrene (5 rings) are known to be, and other PAHs suspected to be, carcinogenic, or teratogenic (Kawahara *et al.* 1995; van Schooten *et al.* 1995; Cerniglia 1992; Wilson and Jones 1992; Liu *et al.* 1992). They constitute significant regulated constituents in creosote-contaminated soils. It should be noted however, that the largest source of PAHs entering the environment is from forest fires; this research focuses on the industrial contamination of soils.

The potential for microbial metabolism as a strategy to remove hydrocarbons from soil is well established, with studies as early as the 1920s (Evans *et al.* 1965). Bioremediation entails the use of microorganisms to metabolize the hydrocarbon contaminant resulting in its removal or attenuation (Wilson and Jones 1992). Bioremediation may lead to the contaminant being completely transformed to biomass, carbon dioxide and water. Bioremediation may also be successful at reducing risk when partial metabolism of the contaminant produces a modified compound that is less toxic, less mobile, "bound" to soil components, or incorporated into soil humus.

Impediments to bioremediation are not always readily correctable. These obstacles may include poor microorganism-contaminant contact due to sorption reactions of the

contaminant to soil solids (Devare and Alexander 1995; Wolfaardt *et al.* 1994), occlusion of the contaminant within stable aggregates (Mott *et al.* 1990), limited oxygen availability, and limited nutrient availability (Pollard *et al.* 1994). Furthermore, characteristics of the contaminated soils themselves may inhibit bioremediation, such as compacted soil structure, a too-low or too-high water holding capacity, and poor aeration, all of which are closely related to soil texture (Song *et al.* 1990). Soils with very high clay contents may exhibit lower rates of microbial activity than soils with moderate to low clay contents.

A simple method for measuring carbon metabolism in soil is to measure the amount of carbon respired as CO_2 (Hinchee and Ong 1992; Heinemeyer *et al.* 1989; Knapp *et al.* 1983). Carbon dioxide is one of the end products of complete metabolism of carbon substrates, along with water and biomass. Although CO_2 evolution does not reflect all of the possible microbial transformations of hydrocarbons in soil, it can provide useful information about the overall microbial activity in the soil. Freijer (1996) stated that several conditions must be met when designing an incubation experiment to study bioremediation of hydrocarbons in soil. Among those listed are: 1) all hydrocarbons are equally degradable; 2) hydrocarbons are the main C source in the system, and ; 3) other reactions involving O_2 and CO_2 are negligible. In addition, the rate of soil respiration may, however, increase in soils following killing of soil microorganisms and removing the toxic substance if the soil originally was substrate-limited. Therefore soil respiration results can be ambiguous in soils containing limited quantities of substrates that are also highly toxic. Care must therefore be taken to avoid conditions of limited supplies of substrates or adding and then removing substrates that are highly toxic to soil microorganisms if soil respiration is to be used to monitor progress of substrate metabolism.

I report here on laboratory respiration measurements aimed at gaining insights into the extent to which soil texture, source of hydrocarbon and prior treatment in a biopile influence the rate of CO_2 evolution by indigenous microorganisms. I hoped also to learn about the rate of response of the indigenous populations of microorganisms in these soils to added amendments or treatments. Four soils were chosen in conjunction with a team of researchers, all investigating components of the bioremediation system from diverse disciplines. Two rates of nutrient amendment were applied to the soils to demonstrate the nutrient demand of the soils. I subjected the soil to freeze-thaw sequences and physical disruption of aggregates to determine if contaminants were protected within aggregates. At the end of the experiment, a simple carbon budget was calculated to relate the amount of carbon respired as CO_2 to the decrease in total DEO.

Materials and Methods

Soils

Four soils were selected for this study (Table 3-1) and were part of a larger project (Rutherford *et al.* 1998). Two creosote-contaminated soils were obtained from sites in Edmonton Alberta and Prince Albert Saskatchewan. Two oil-contaminated soils were obtained from sites in Devon Alberta and Montreal Quebec. All four soils were contaminated industrially, and the contamination occurred between 10 and 50 yr ago. The Montreal soil, which had previously been biotreated, was included for study because it still contained recoverable DEO.

Treatments

Four treatments were applied in a randomized complete block design with three replicates. The treatments were: incubated control (no additional nutrients); normal nutrient rate (addition of N:P:S at ratios of 10:1:1); and, high nutrient rate (twice the normal rate). The nutrients were added as NH_4NO_3, K_2SO_4, KH_2PO_4, and K_2HPO_4 dissolved in water. Quantities of each soil were reserved and stored at 4° C as a non-amended, non-incubated control. The amount of nitrogen added to each soil was based on the amount of extractable hydrocarbon in each soil (Eq. 3-1; McGill 1975).

$$gNg^{-1}soil = \frac{[(190 \times \%DEO - C) - 140]}{2 \times 10^6} \qquad \text{[Eq. 3-1]}$$

Incubation Conditions

Samples of 50 g (oven-dry equivalent) of soil were placed in 250-mL Erlenmeyer flasks. The soil in each flask was maintained at 50% of the water holding capacity (WHC) of the soil (water content after 3 h drainage), determined by the funnel method (Harding and Ross 1964). The funnel method entails the saturation of a known amount of soil in a pre-weighed glass funnel plugged with glass wool, with the funnel sitting in a beaker of water overnight. The funnel is then raised out of the water and the soil sample allowed to drain for 3 h. The funnel containing the drained soil sample is then transferred to an empty beaker and then dried overnight at 105°C (or to constant weight). The water holding capacity was calculated on the basis of clean, oven-dry soil weight. The hydrophobic nature of the contaminants slowed water absorption into the soils, but did not prevent it.

The soils were incubated at ~40% WHC for 7 d prior to adding the nutrient solution. The addition of the nutrient solution marked the start of the experiment and at this time the soils were all brought to 50% WHC by weight. The incubation flasks were plugged with foam to permit air circulation and prevent contamination with airborne particles. The flasks were incubated in the dark for 10 wk at 22 ± 2°C.

Carbon Dioxide Production

Carbon dioxide production rates were determined by sealing each flask with a Suba Seal, immediately measuring the concentration of the gas in the head space, waiting 2 h, and then measuring the accumulation of CO_2 in each sealed flask. Concentration of CO_2 was measured using a Hewlett Packard 5890 Series II gas chromatograph with a 20 cm Hayesep Q column and a helium carrier gas flow rate of 30 mL min^{-1}. A Hewlett Packard 3396 Series II integrator recorded the thermal conductivity detector signals and calculated the areas under the peaks. The difference in CO_2 concentration values (ppm) was divided by the oven dry mass of soil and by the time interval between readings (h) to report ppm CO_2 g^{-1} soil h^{-1}. This value was converted to μg CO_2-C g^{-1} soil h^{-1} using the following equation:

$$\mu g\,C = \left(\frac{\mu L\,CO_2}{\text{L } flask\ vol}\right) \times \left(\frac{flask\ vol\ \text{L} - }{soil\ particle\ vol\ \text{L}}\right) \times \left(\frac{12\,\mu g\,C}{\mu mol\,CO_2}\right) \times \left(\frac{1\,\mu mol\,CO_2}{22.4\,\mu L^{*}}\right) \qquad \text{[Eq. 3-2]}$$

*value adjusted for atmospheric pressure and temperature readings for each day

DEO-C

Soil (10 g) mixed with an approximately equal amount of $MgSO_4$ (drying agent) was Soxhlet-extracted overnight with 200 mL dichloromethane at a rate of 4 cycles h^{-1}. The solvent was distilled under vacuum until < 5 mL remained and the residual solvent and extracted organics were transferred to a 25-mL volumetric flask and brought to volume with dichloromethane. A 10-mL subsample was transferred into an aluminum weighing dish and the solvent allowed to volatilize, leaving behind a tarry residue (DEO) which was weighed on a precision balance (4 decimal places). The residue was analyzed for carbon, hydrogen, oxygen, and nitrogen contents by microanalysis on a Carlo Erba CHNS-O Elemental Analyzer EA1108 in the Microanalytical lab of the Department of Chemistry. The remainder of the extract was stored in 20-mL glass scintillation vials for subsequent PAH analysis by GC and mass spectrometry.

The PAH analyses were performed by M. Rawluk at the Alberta Research Council (Edmonton Canada) on a Carlo Erba 5160 HRGC connected to a Finnigan 4500 MS with multiple ion detection. The HRGC used a Restik 30 m XTI-5 fused silica column and helium (35 cm s^{-1}) as the carrier gas. These analyses separated and quantified naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene, and chrysene.

Total Soil Carbon

Total soil carbon was determined by oxidizing 0.5-g soil samples in a Leco CR12 high frequency induction furnace. Evolved CO_2 was quantified using infrared absorption and the C content expressed on a dry soil basis.

Carbonates were determined by the titrimetic method of Bundy and Bremner (1972). Samples of 0.5-1.0 g soil were weighed into 250-mL wide-mouth Erlenmeyer flasks, a drop of *n*-octyl alcohol was added, and then the flask was stoppered with a modified rubber stopper. Having a glass tube pass through it so that within the flask, a small vial containing 5 mL of 2 M KOH to trap CO_2 was suspended above the soil sample, and above the stopper, the tube was sealed with a septum. A 50-mL syringe was used to withdraw ~150 mL of air from the flask through the septum. Another 50-mL syringe was used to inject 20 mL of 2-M HCl into the flask. The flask was gently swirled and then allowed to equilibrate for 24 h. After 24 h had passed, the KOH trap was quantitatively transferred to a clean 125 mL Erlenmeyer flask and brought to a volume of 50 mL with distilled, deionized water. A few drops of phenolphthalein indicator were added and the solution titrated with 0.4 M HCl until the colour began to fade. A 0.1 M standardized HCl solution was then used to titrate the solution just to the clear endpoint. A few drops of bromocresol green solution were then added and the solution titrated with the 0.1 M HCl until a bright yellow end point was reached.

$$\%CaCO_3\ equivalents = \frac{S - B}{mass\ of\ soil\ sample(g)} \qquad \text{[Eq. 3-3]}$$

where:

S = mL of 0.1 M HCl required to titrate sample from the phenolphthalein endpoint to the bromocresol green end point.

B = mL of 0.1 M HCl required to titrate blank from the phenolphthalein endpoint to the bromocresol green end point.

Water-Stable Aggregates

Wet aggregate stability was determined using five nested sieves (0.125 mm, 0.25 mm, 0.5 mm, 1 mm, and 2 mm). Each sample was wet sieved, collected, and weighed according to the method described by Angers and Mehuys (1993). Water-stable aggregates (WSA) are the proportion of aggregates of a given size (corresponding to the sieve sizes) that do not disintegrate under the influence of water disruption. Mean weight diameter was calculated as:

$$MWD = \sum_{i=1}^{n} x_i \times WSA_i \qquad \text{[Eq. 3-4]}$$

where i=1, 2, 3…n, and corresponds to each fraction collected,
x_i= the mean diameter of each size fraction, and
WSA_i=is as described above

Statistics

One-way ANOVAs were applied to the DEO-C, total soil C, and water stable aggregate data. An LSD means separation was used to determine significant differences within these data. The PAH analyses were not replicated.

A nonparametric 1-way procedure (PROC NPAR1WAY) was used to compare the time course of CO_2 evolution rates among treatments within a soil. A χ^2 test was used to compare the sum of scores for each line and determine significant differences among treatments.

Nonlinear analysis (PROC NLIN) was used to fit three kinetic models (logistic, Gompertz, and exponential) to the CO_2 evolution data and to estimate the parameter values for each model. To indicate the proportion of the variation explained by the model, the coefficient of multiple determination (R^2) was calculated (Ellert and Bettany 1992):

$$R^2 = 1 - \left(\frac{RSS}{corrected\ total\ RSS} \right) \qquad \text{[Eq. 3-5]}$$

where RSS is the residual sum of squares. To discriminate between models if more than one model fit the data, pairwise F-tests were applied as described by Ellert and Bettany (1992):

$$F = \frac{\left(\frac{|RSS_1 - RSS_2|}{(p_2 - p_1)} \right)}{\left(\frac{RSS_2}{n - p_2} \right)} \qquad \text{[Eq. 3-6]}$$

where:

RSS_1 = residual sum of squares for model 1 (the simpler model)
RSS_2 = residual sum of squares for model 2 (the more complex model)
n = number of data points used
p_1 = the number of parameters in the simpler model
p_2 = the number of parameters in the more complex model

The calculated F value is calculated at $F(a, p_2-p_1, n-p_2)$ where a is the desired level of statistical significance.

Results

The rate of CO_2 evolution in the nutrient-amended Devon soil samples increased significantly ($p<0.05$) over that of the non-amended control within 48 h of nutrient addition (Fig. 3-2). The rate returned to the level of the control treatment at 600 h of incubation. The maximum CO_2 evolution rate attained in the normal nutrient amendment was somewhat lower than that of the high nutrient treatment. The rate of CO_2 evolution from the Montreal soil samples (Fig. 3-2) was very low throughout the incubation, and no significant differences in CO_2 production rates were detected between the control and either nutrient-amended treatment. A spike is seen in the curves for the Montreal soil between 282 and 320 h, during which time the samples were treated with glucose (1 mg g^{-1} soil) to test the possibility that the soils had been rendered sterile by the contamination. The soils actively respired and the CO_2 evolution rates returned to the original baseline in less than 38 h, indicating that the glucose was consumed.

The CO_2 evolution rates of the amended treatments in both creosote-contaminated soils increased significantly ($p<0.05$) over the evolution rates in the controls within 48 h of nutrient amendment (Fig. 3-3). These peaks returned to the baseline of the non-amended control within 260 h for the Edmonton samples and within 550 h for the Prince Albert samples.

The soils were frozen between 710 and 875 hours to introduce a freeze-thaw cycle to disrupt soil aggregates. I hypothesized that aggregates may protect contaminants. Increased CO_2 evolution rates ranged from about 1.25 μg CO_2-C g^{-1} soil h^{-1} for the Montreal soil to about 15 μg CO_2-C g^{-1} soil h^{-1} for the Devon soil.

At 1400 h, the Devon, Edmonton, and Montreal soil samples were agitated and stirred with a glass rod, in an attempt to simulate the aggregate destruction of cultivation and to expose aggregate-protected contaminant. A 0.5 μg CO_2-C g^{-1} soil h^{-1} increase was measured in the CO_2 evolution rate for all three treatments but only in the Devon soils.

The Prince Albert samples were weakly aggregated, due to the high sand content of this soil, and few aggregates were present for stirring to disrupt. A calculation of C metabolized to this point from the Prince Albert treatments (normal nutrient amendment: 1800 μg C g^{-1} soil, high nutrient amendment: 1900 μg C g^{-1} soil) was related to the amount of nitrogen added at the beginning of the incubation. This calculation (Eq. 3-4) used a carbon utilization efficiency of 50%. The resulting $C_{metabolized}$:N_{added} ratios were 19.5:1 (normal nutrient amendment) and 35:1 (high nutrient amendment).

$$C_{metabolized} : N_{added} = \frac{R}{CUE \times N_{add}} \qquad \text{[Eq. 3-7]}$$

where:

R = cumulative CO_2-C respired over 1400 h (mg C g^{-1} soil)
CUE = carbon utilization efficiency (fraction)
N_{add} = quantity of N added to the soil at the start of the incubation (mg N g^{-1} soil)

Because these ratios are much higher than the 10:1 considered optimal for microbial growth and metabolism, a second nutrient addition, based on the calculated amount of remaining hydrocarbon (Eq. 3-1), was applied to the Prince Albert nutrient-amended soil

samples. A significant ($p<0.05$) increase in CO_2 evolution rate of 4 μg CO_2-C g^{-1} soil h^{-1} was measured following this addition.

Table 3-2 summarizes the carbon disposition in the non-amended- and high nutrient-amended treatments for all four soils after incubation. The total soil carbon decreased significantly ($p<0.05$) in both nutrient-amended treatments in the Devon, Edmonton, and Prince Albert soil samples, compared with the non-incubated reserved samples. Only in the high-rate nutrient-amended Edmonton treatments was this loss not significantly greater than the quantity of CO_2-C lost from the normal-rate amended treatment. The total soil C in the Montreal soil samples did not change during the incubation. In all four soils the DEO-C fraction appeared to decrease compared with the non-incubated reserved samples, however only in the Devon and Montreal soils was this decrease statistically significant. This decrease was, in all treatments, greater than the quantity of C lost as CO_2 from that treatment.

The PAH contents of the two creosote-contaminated soils appeared to decrease following the incubation (Table 3-3). In the high nutrient-amended Edmonton samples, losses ranged from 28% (pyrene) to 99% (phenanthrene). Losses of PAHs in the high nutrient-amended Prince Albert samples ranged from 4% (chrysene) to 99% (naphthalene). In general, greater losses were observed from the lower molecular weight PAHs. Interestingly, more PAH-C (total quantity of C present in PAHs) was lost from the Prince Albert soil (3440 μg g^{-1} soil) than from the Edmonton soil (1140 μg g^{-1} soil), even though both soils lost 74% of their total PAHs. Moreover, the amount of PAH-C that disappeared from each soil during the incubation was twice the amount of C lost as CO_2 from the Edmonton soil and 1.5-times the amount of CO_2-C evolved from the Prince Albert soil.

The elemental ratios, C:N, C:O, and C:H were calculated for the DEO of each treatment at the end of the incubation (Table 3-4). These ratios remained constant within each soil. The C:H ratios were not significantly different among all four soils.

No significant differences in MWD of aggregates were detected among treatments within the Devon, Edmonton, or Prince Albert soils. The soil with the most clay, Montreal, had the highest MWDs, whereas the soil with the lowest clay content, Prince Albert, had the lowest MWDs.

Three non-linear models were fitted against the incremental CO_2 production data for each soil. In order to declare a model suitable for a soil, the model had to converge (fit the data) for all three treatments within the soil. The three models examined were the exponential growth model (monomolecular growth), the logistic model, and the Gompertz model. The logistic model was rejected for all four soils because the model would not converge for all of the three treatments for any of the soils.

In the exponential model, the limiting component is the substrate availability (France and Thornley 1984). The growth rate (dC/dt) is proportional to the amount of available substrate (S) and growth is irreversible. In this model, mineralization begins quickly with little lag time between addition of substrate and evolution of substrate-derived CO_2. The differential form of the exponential model is:

$$\frac{dC}{dt} = kS \qquad \text{[Eq. 3-8]}$$

In order to fit this model to incremental CO_2-C production data, the form used is:

$$C_{it} = C_o\left(e^{-kt}\right)\left(e^{kt} - 1\right) \qquad \text{[Eq. 3-9]}$$

where:

C_0 = potentially mineralizable C (μg)

k= specific mineralization rate (h^{-1})

C_{it} = quantity of C evolved as CO_2 during interval i preceding t.

The logistic growth model is similar to the exponential model, but differs in that the limiting components are both the organisms and substrate. Again, the growth rate is proportional to the amount of substrate and growth is irreversible. The logistic growth model may be represented as:

$$\frac{dC}{dt} = kC(C_f - C) \qquad \text{[Eq. 3-10]}$$

To apply this model to incremental data, the following equation is used:

$$C_i = C_o\left[\frac{1}{1+\left(\frac{C_o}{x}-1\right)e^{-kt}} - \frac{1}{1+\left(\frac{C_o}{x}-1\right)e^{-k(t-i)}}\right] \qquad \text{[Eq. 3-11]}$$

where:

C_i = CO_2-C production during interval "i" (μg)

C_f = CO_2-C produced at time "t" (μg)

C_o = potentially mineralizable C (μg)

k = rate constant (h^{-1})

x = initial quantity of mineralizable C (μg)

t = time (h)

i = interval between measurements

The limiting components of the Gompertz model are the organisms and their growth rate. Substrate is not a limiting factor in this model. As well this model also includes a factor to describe diminishing degrees of growth. This model allows for a longer lag time between substrate addition and substrate mineralization. The differential form of the model is:

$$\frac{dC}{dt} = hCe^{-Dt} \qquad \text{[Eq. 3-12]}$$

where:

C = CO_2-C production

h = first order rate constant ($time^{-1}$)

D = parameter describing the reduction of the specific growth rate ($time^{-1}$)

The incremental form of the model, as modified by Ellert and Bettany (1992) is:

$$C_i = C_o e^{h\left[1-e^{-k(t-i)}\right]} - C_o e^{h\left(1-e^{kt}\right)} \qquad \text{[Eq. 3-13]}$$

where:

C_i = incremental quantity of CO_2-C evolved (μg)

C_0 = potentially mineralizable C (μg)

h = a proportionality constant (unitless)

k = acclimatization coefficient defining the increase in α through time (d^{-1})

In all models C_0 may be analogous to potentially mineralizable C. Direct measurements of CO_2-C can then be used to fit the models to the data sets.

Only the exponential model was able to fit the CO_2 data obtained for all three treatments of all four soils. The R^2 values are fairly low for some treatments, particularly the control and normal nutrient amendment treatments of the Montreal soil (R^2<0.55). Figure 3-7 shows examples of how well the model fit the data for the Devon soil and for the Edmonton soil.

Discussion

The rapid increase in respiration rates of the nutrient-amended Devon, Edmonton, and Prince Albert soil samples, over the rates of their non-amended controls, is evidence that successful bioremediation of these soils is limited, at least in part, by nutrient availability. The lack of an increase in CO_2 evolution following nutrient addition in the Montreal soil samples raises several questions regarding the physical, chemical, and biological status of the soil. A small amount of glucose added to this soil was quickly metabolized and detected as evolved CO_2, evidence that the Montreal soil was not sterile.

There was little production of CO_2 in the Montreal samples. The Montreal soil had previously been "treated," yet still contained 0.8% recoverable DEO. It may be that 0.8% is a threshold below which no further biodegradation is possible. The electrical conductivity of the Montreal soil, 4.95 dS m^{-1}, is greater than 4 dS m^{-1}, the value above which soils are considered "salt-affected." The high salt content may inhibit microbial metabolism of the hydrocarbons, although it did not hinder metabolism of the added glucose. Therefore, unless a hydrocarbon-specific suite of microorganisms was selectively inhibited, I can conclude that inhibition by salinity was not likely. The soil is a clay loam, and the aggregates <2 mm (diameter) are very stable. This physical characteristic may contribute to the occlusion of contaminant in aggregates within interior micropores too small to be microbial habitats. The rapid metabolism of the added glucose was evidence that aeration was not limiting in the Montreal soil samples. This soil is characterized by high clay content, water-stable aggregation, retention of up to 8 mg DEO g^{-1} soil and although viable, the microbial community exhibits little activity unless exogenous substrate is added.

The soil samples having the least clay, the Prince Albert samples, were the quickest to respire increased quantities of CO_2 following nutrient amendment and responded to the greatest degree, reaching a peak nearly four times greater than that achieved by the Devon or Edmonton soils. As well, this light-textured soil had the greatest percentage of the DEO-C loss as CO_2-C (72%). This may be due in part to the lack of aggregation allowing a greater degree of contact between microorganisms and the hydrocarbon contaminants. It may be concluded that those soils having large quantities of clay, and that are strongly aggregated are not as well suited to passive bioremediation strategies that rely on removing the contaminant through complete oxidation. The percent of the DEO-C loss that can be accounted for as evolved CO_2-C, in order of decreasing clay content was Edmonton (25%), Montreal (9%), Devon (47%). The heavier-textured soils appear to have a greater degree of C transformation out of the DEO fraction, within the soil C system. This poses further questions regarding the mechanisms of this transformation,

whether due to interactions with soil minerals, or due to alternate metabolic pathways driven by the physical limitations imposed by heavier-textured soils.

Since there was no significant difference among neither treatments nor soils in the amount of CO_2 evolved during thawing, I speculate that the CO_2 evolved during this process was due to the release of the atmospheric CO_2 that was trapped in air pockets as the soil solution froze.

Stirring resulted in a small increase in the production of CO_2 in the Devon samples. During stirring it was observed that the aggregates were easily broken in the Devon soil samples, possibly exposing protected hydrocarbons. The Edmonton and Montreal samples both had aggregates that were stronger, and more difficult to break during stirring. These aggregates did not fracture readily, and therefore little fresh contaminant could be exposed. Note that the MWD of the Edmonton soil samples is not different from that of the Devon soil samples, yet it was observed that the resistance of the aggregates to stirring was greater in the Edmonton samples.

At this same time (1400 h), a second nutrient amendment to the Prince Albert samples was followed by increased CO_2 evolution rates. This demonstrates the continuous nutrient demand of bioremediation.

I move now to comment on the redistribution of C within these soils during incubation. A simplified model of soil carbon to illustrate some possible redistributions of DEO-C in contaminated soils (Fig. 3-6) is presented.

The decrease in DEO-C is greater than the loss of CO_2-C in the Devon, Prince Albert, Edmonton, and Montreal soil samples (high amendment). The quantity of carbon evolved as CO_2, can account for the decrease in total soil carbon, within limits of analytical variability given analytical methods used for each fraction. I infer from this that the DEO-C was transformed within the soil C system rather than oxidized to CO_2. I hypothesize that DEO-C was transformed into the conventional SOM-C fraction, part of which is biomass. Although the Montreal samples respired little throughout the incubation, they showed evidence of substantial transformations of the DEO-C within the soil carbon system. I cannot say that successful bioremediation was inhibited in this soil, merely that total metabolism of the hydrocarbons to CO_2 and H_2O was not occurring. Freijer (1996) reported similar results when hydrocarbons were incubated in soil systems, attributing 80% of the decrease in hydrocarbon concentration in soil to mineralization to CO_2, and concluding that 20% of the decrease was due to transformation into biomass and metabolites. This transformation of substrate C into more stable, organic forms is commonly seen when radiolabeled organic amendments, such as ^{14}C-labeled wheat straw (Cogle *et al.* 1989) or ^{14}C-glucose (Ladd *et al.* 1992) are added to soil. Goodin and Webber (1995) demonstrated that portions of ^{14}C-anthracene were rendered non-extractable, and therefore hypothesized to be bound residues, after 21 wk of incubation in soil. The amount of anthracene retained as bound residues ranged from ~35-75 % of the total amount added, depending on the initial quantity added and the soil type.

The PAHs in the creosote-contaminant soils decreased throughout the course of the incubation. The lower molecular weight PAHs disappeared to the greatest extent. Naphthalene is volatile and it is possible that its removal from the soil may have been partly or entirely abiotic. The Edmonton soil showed substantial removal of the more complex pyrene, benzo[a]anthracene, and chrysene, ranging from 25-30%. Removal of

PAHs from soil is essential, as these are known health hazards. Interestingly, the Prince Albert soil samples, which respired more total C than did the Edmonton samples, lost a smaller proportion of its total PAHs than the Edmonton soil. This suggests that the PAH components are lost preferentially in the Edmonton soil, however some other component of the contamination is lost preferentially from the Prince Albert soil.

The observed "sequential" use of specific PAHs is not a novel idea in bioremediation. Results published by Trzesicka-Mlynarz and Ward (1995) and Bossert and Bartha (1986) were similar to mine, as they too reported a greater rate of disappearance of the lower molecular weight PAHs compared with those having four or more rings. Studying aqueous contaminated systems, Rosenberg *et al.* (1992) postulated that "droplets" of hydrocarbons are sequentially colonized by increasingly more specific microorganisms. Those microbes capable of metabolizing the simpler components are more numerous. As simple compounds are reduced in quantity, more highly specialized microorganisms are able to colonize the droplet and use more complex compounds as substrate. It may be that a similar dynamic is functioning in contaminated soil systems.

Protection of contaminant within the stable aggregates of heavier-textured soils may also be responsible for decreased oxidation of contaminants, however the intensity of the Soxhlet procedure with dichloromethane allows this contaminant fraction to be accounted for as DEO.

The most important idea to be generated by this investigation must be clearly stated: In some soils, contaminant attenuation by incorporation into soil organic matter may be substantial. Partial metabolism of contaminant molecules may produce sufficiently modified hydrocarbons that are "bound" to organic matter functional groups, or that may "dissolve" into the SOM. In this event, contaminants may not have been removed from the soil system in the conventional manner, but may still be non-retrievable, diminishing the threat to the environment posed by the original compound. The generation of CO_2 in three of the soils studied, in conjunction with greater quantities of C lost from the DEO fraction suggests that transformations of the contaminants are occurring in these soils. This hypothesis merits further study to determine the mechanism of C redistribution.

Table 3-1. Characteristics of hydrocarbon-contaminated soils from Devon, Edmonton, Prince Albert, and Montreal used for incubations.

Soil	pH	EC[1]	Total C	CO_3^{-2}-C	DEO-C[2]	WHC[3]	Texture[4]
		($dS\ m^{-1}$)		$mg\ g^{-1}$ soil		(%)	
Devon	7.7	0.34	27.7	0	18.1	48	Loam
Edmonton	7.3	1.25	37.9	8.3	10.7	52	Silty clay
Prince Albert	8.1	0.31	20.6	11.9	10.1	29	Sand
Montreal	7.5	4.95	35.1	30.3	8.1	41	Clay loam

[1]Electrical Conductivity
[2]Dichloromethane Extractable Organic Carbon
[3]Water holding capacity
[4]Rutherford *et al.* 1998

Table 3-2. Soil carbon changes measured following 10 wk incubation. Calculated differences significant unless otherwise noted ($p<0.05$).

Soil	Total Soil C			DEO-C			CO_2-C Evolved[1]
				$mg\ C\ g^{-1}$ soil			
	Non-incubated	High N	Change	Non-incubated	High N	Change	
Devon	27.7	25.6	-2.1	18.1	13.02	-5.11	2.39
Edmonton	37.9	37.0	-0.9	10.7	8.44	-2.22[2]	0.56
Prince Albert	20.6	18.2	-2.4	10.1	6.88	-3.21[2]	2.33
Montreal	35.1	35.1	0	8.1	6.40	-1.65	0.15

[1]cumulative
[2]calculated difference not significant

Table 3-3. Loss of selected PAHs in the high nitrogen amendment of the creosote-contaminated soils.

	Soils					
	Edmonton			Prince Albert		
	PAH concentration ($\mu g\ g^{-1}$ soil)			PAH concentration ($\mu g\ g^{-1}$ soil)		
PAH	t=0	t=10 wk	% Loss	t=0	t=10 wk	% Loss
Naphthalene	10.8	1.3	88.4	>2175	1.5	99.9
Acenaphthylene	1.25	*	100	2.25	1.5	33.3
Acenaphthene	186	*	100	385	182	52.9
Fluorene	175	*	100	330	121	63.3
Phenanthrene	473	2.5	99.5	775	23.8	96.9
Fluoranthene	360	128	64.4	470	355	24.5
Pyrene	293	208	28.6	418	320	23.3
Benzo[a]anthracene	69	46.0	33.3	187	135	28.1
Benzo[a]pyrene	68	34.5	49.5	156	149	4.0

*Below detection limits

Table 3-4. Mean element concentrations (C, N, O, H) of the DEO for all four soils.

Soil	%C (s.d.[1])	%N	%O	%H
Devon	86.0 (0.13)	0.19 (0.01)	1.08 (0.13)	12.1 (0.22)
Montreal	84.6 (0.50)	0.36 (0.03)	3.25 (0.31)	9.97 (0.17)
Edmonton	84.2 (0.98)	0.25 (0.07)	3.56 (1.43)	9.21 (0.28)
Prince Albert	88.1 (0.69)	0.57 (0.08)	2.42 (0.35)	7.63 (0.24)

[1]Standard deviation

Table 3-5. Exponential model parameters calculated for each soil-treatment combination.

Soil	Treatment	Model Parameters		R^2
		C_0 ($\mu g\ g^{-1}$ soil)	k (h^{-1})	
Devon	non-amended	2978 ± 294	0.000755 ± 0.00012	0.76
	normal	2963 ± 265	0.000882 ± 0.00014	0.70
	high	3039 ± 204	0.00106 ± 0.00014	0.83
Montreal	non-amended	171 ± 29	0.000998 ± 0.00027	0.49
	normal	179 ± 24	0.00115 ± 0.00025	0.55
	high	96 ± 18	0.002451 ± 0.00074	0.90
Edmonton	non-amended	507 ± 33	0.00134 ± 0.00014	0.92
	normal	447 ± 65	0.00350 ± 0.00076	0.73
	high	495 ± 92	0.00529 ± 0.00144	0.68
Prince Albert	non-amended	1157 ± 92	0.00126 ± 0.00016	0.91
	normal	1890 ± 267	0.00340 ± 0.00073	0.74
	high	1953 ± 340	0.00455 ± 0.00117	0.66

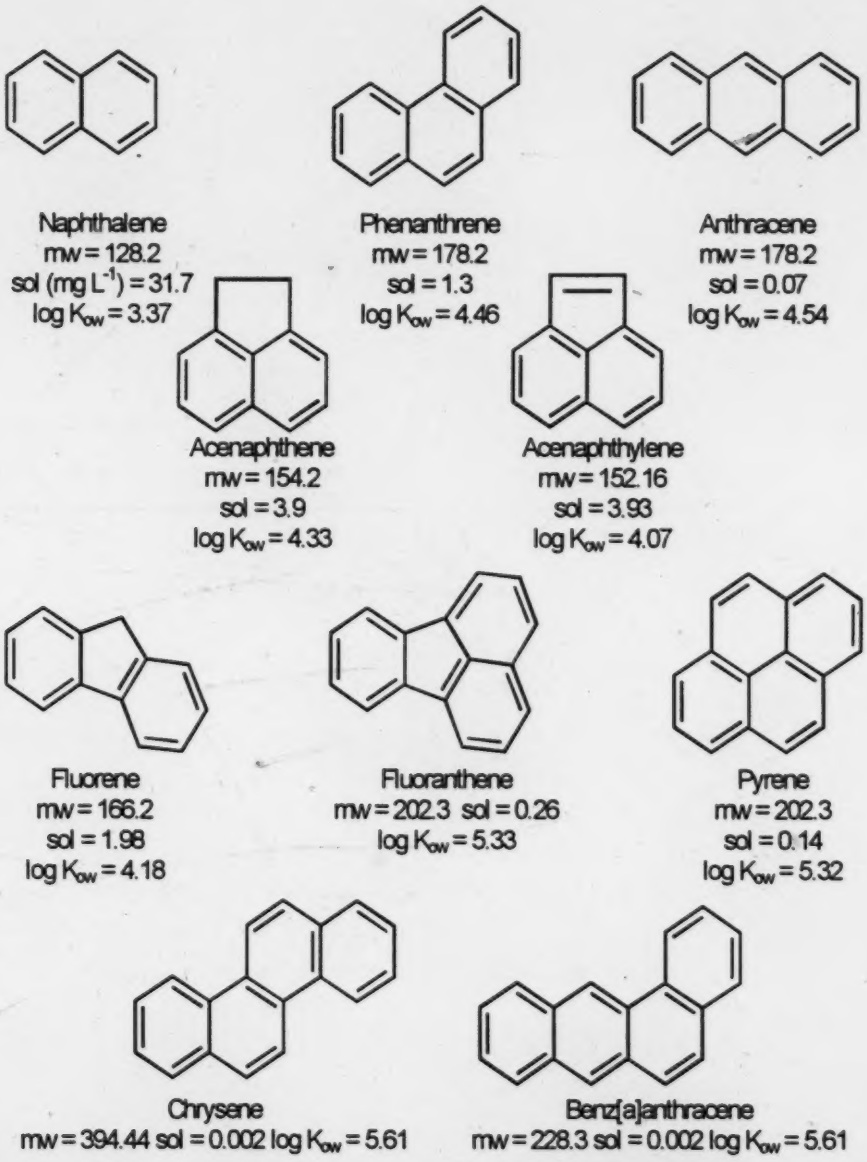

Figure 3-1. Some polycyclic aromatic hydrocarbons (PAHs); all are EPA Priority Pollutants (Wilson and Jones 1993; constants from Sims and Overcash 1983).

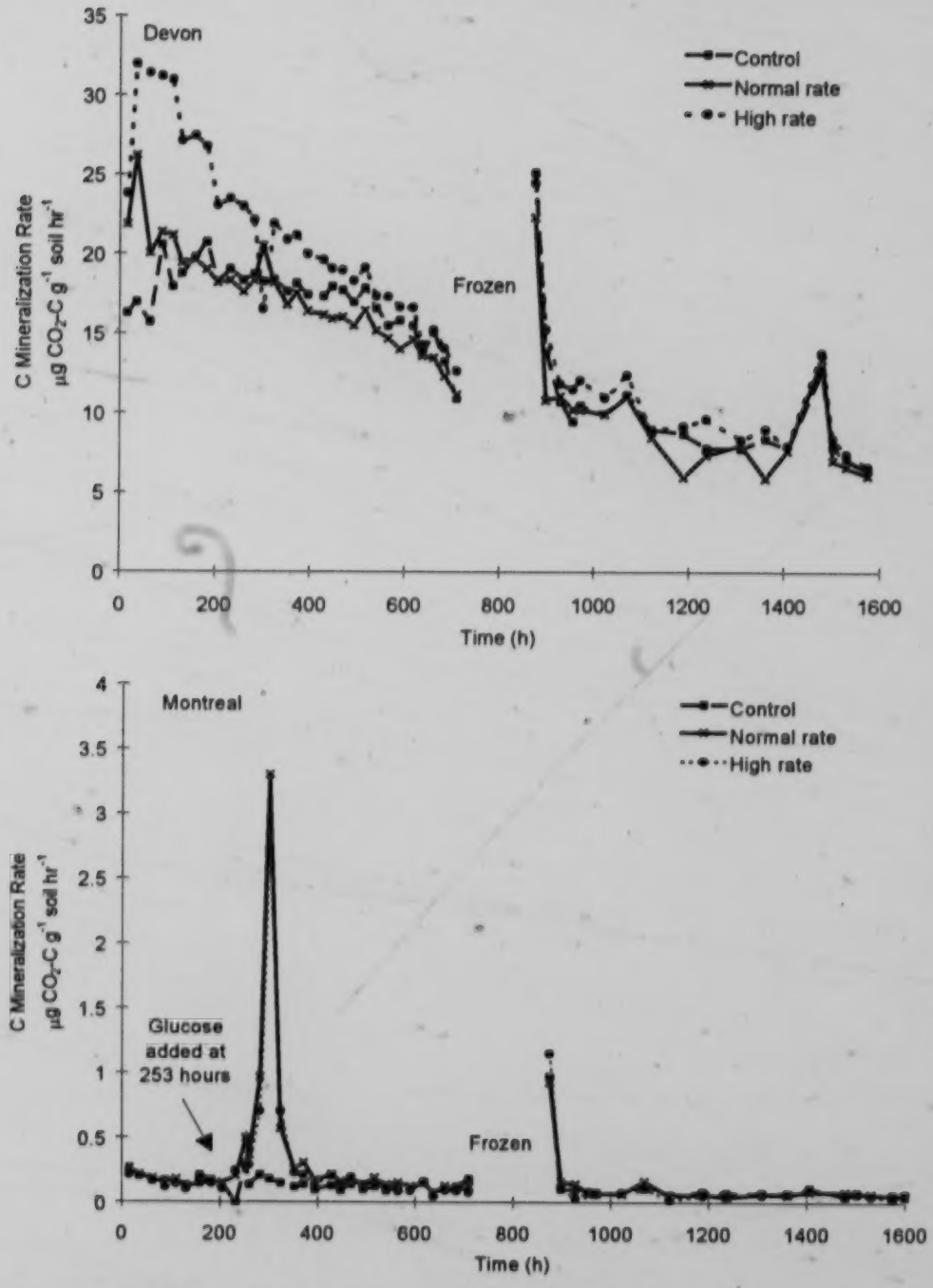

Figure 3-2. CO_2-C evolution rates in two oil-contaminated soils; treatments are rates of nutrient amendment.

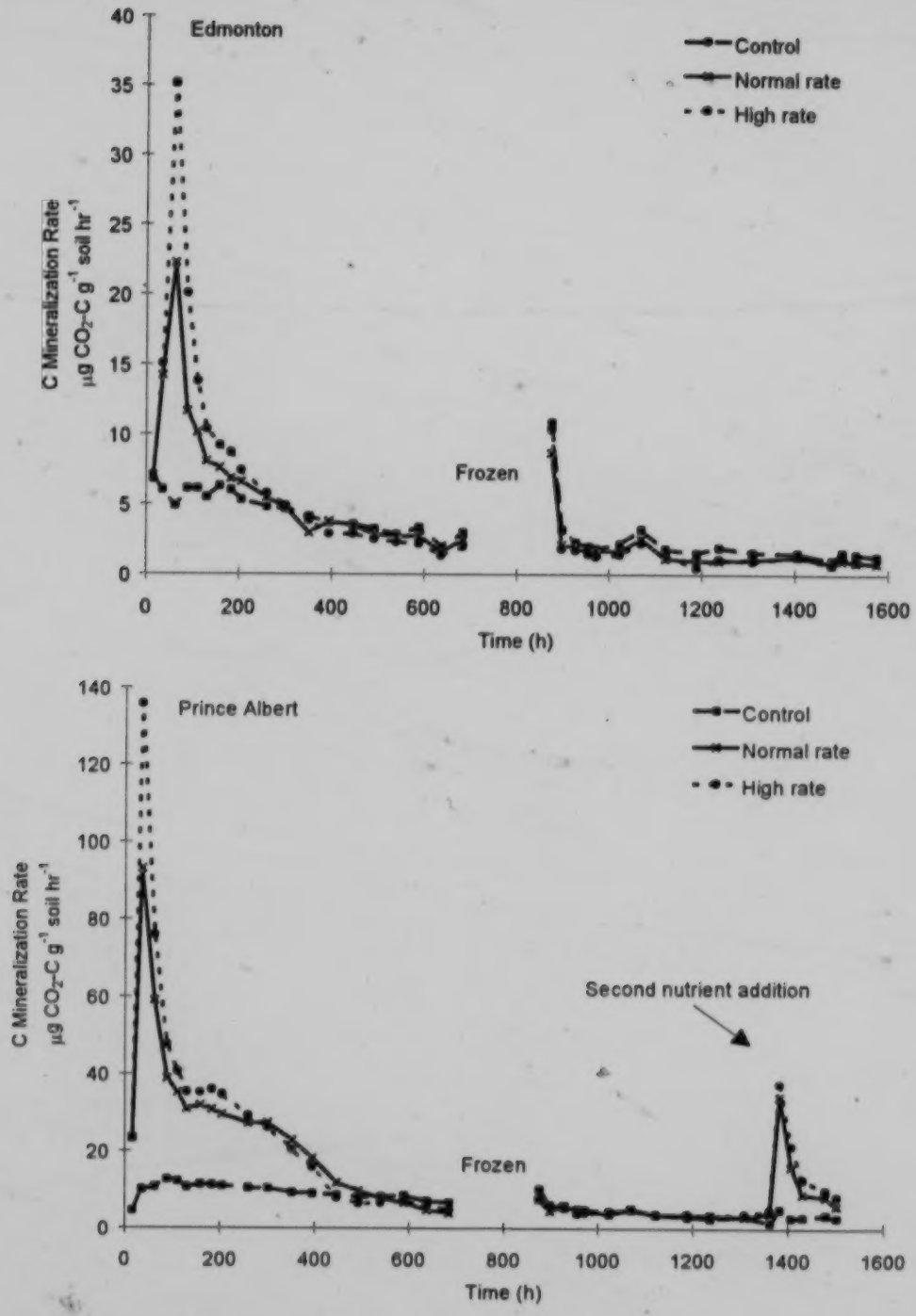

Figure 3-3. CO_2-C evolution rates in two creosote-contaminated soils; treatments are rates of nutrient amendment.

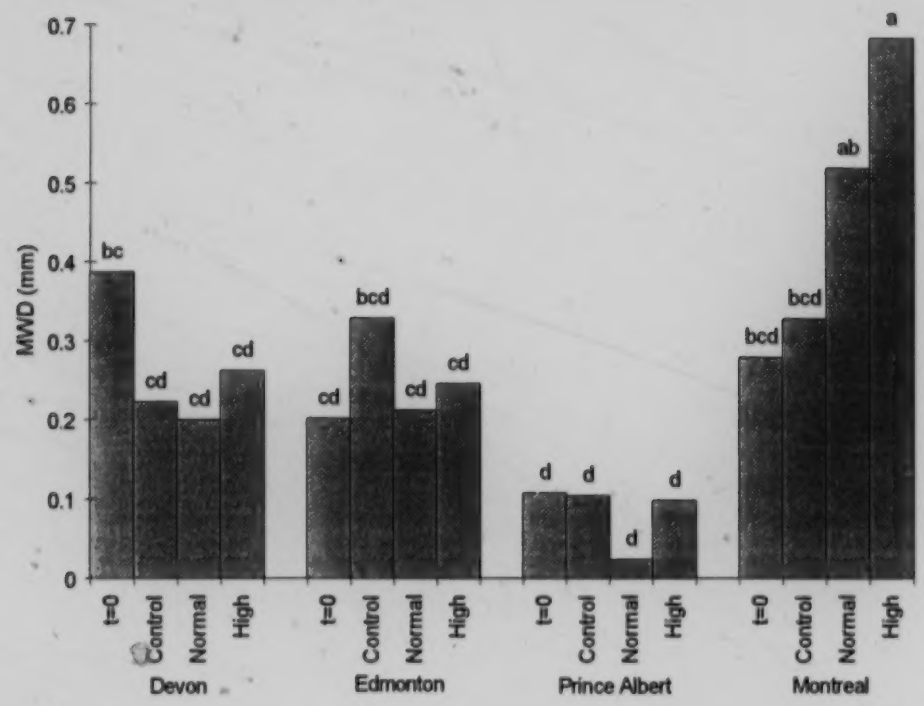

Figure 3-4. Mean weight diameters for all three treatments of each soil and the non-incubated samples. Bars indicated with the same letter are not significantly different ($p<0.05$).

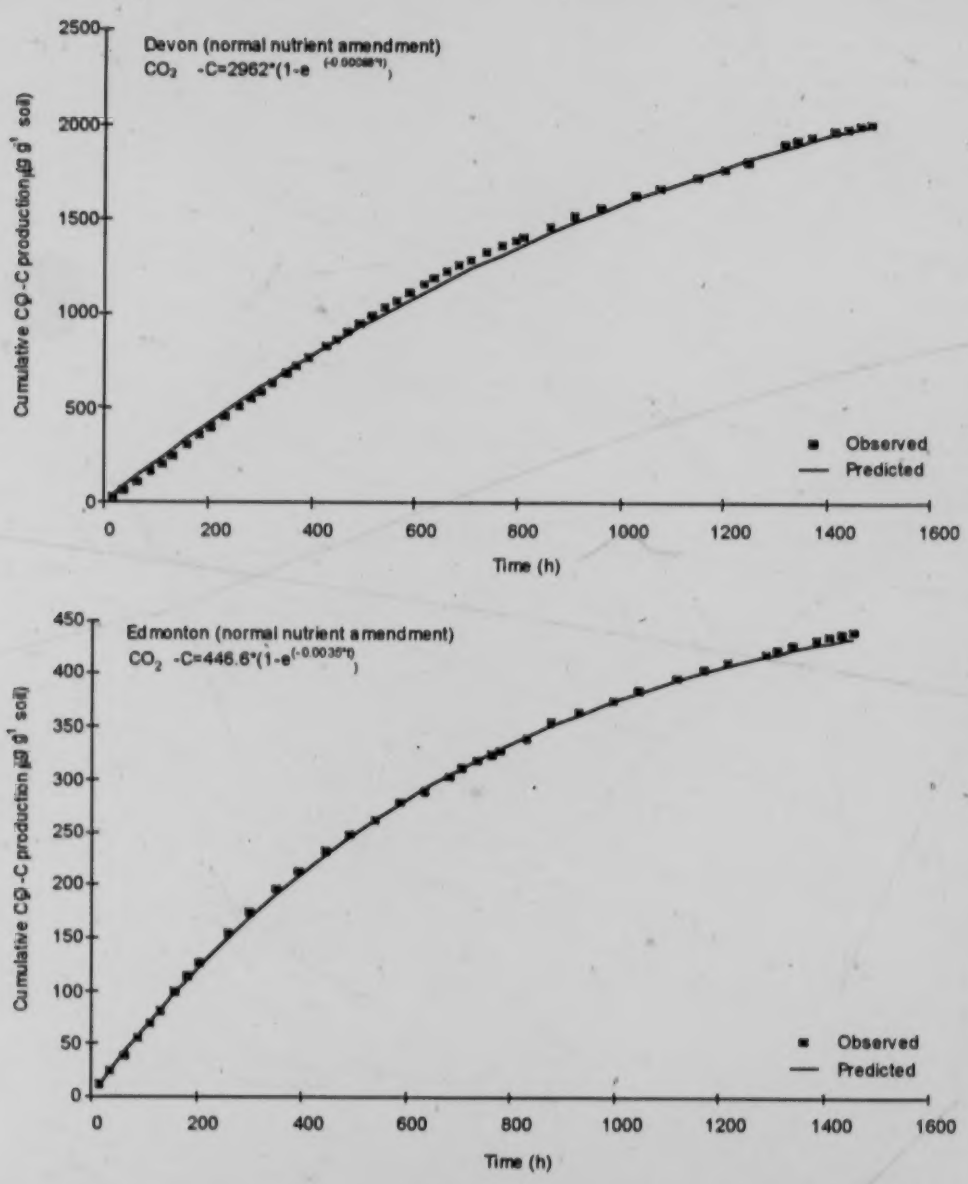

Figure 3-5. Examples of fitting the exponential model to the CO_2 production data for the Devon (oil-contaminated) and Edmonton (creosote-contaminated) soils. Though incremental data were used, the example presented shows the cumulative form.

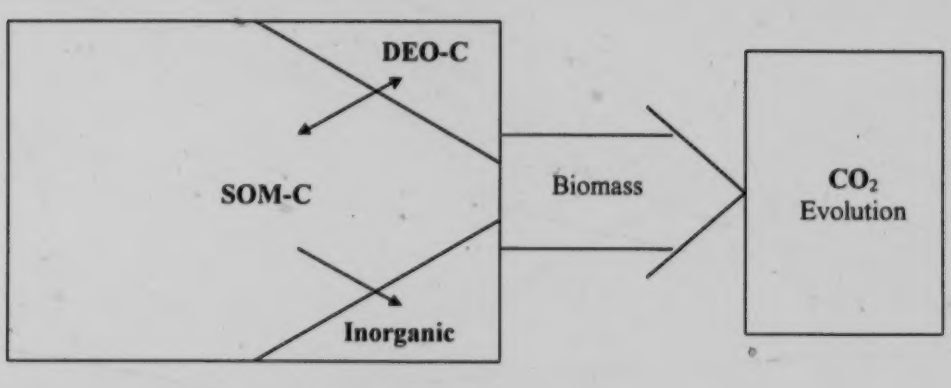

Figure 3-6. A simplified model of C transformations in soil.

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CHAPTER 4

FATE OF RADIOLABELED MODEL ORGANIC CONTAMINANTS IN TWO WEATHERED CONTAMINATED SOILS

Introduction

The study of hydrocarbon-contaminated soils has produced a rich literature describing the metabolic transformations of contaminant-C in soils and their potential as biotreatment mechanisms. Much of the research has focused on either synthetically or freshly contaminated soil systems. With little research involving soils in which the contamination has aged ("weathered contaminated soils") (Dechênes *et al.* 1996; Yeom *et al.* 1996), it is widely acknowledged that the recently prepared systems are incomplete analogues for the aged contaminated soils (Hatzinger and Alexander 1995; Pavlostathis and Mathavan 1992). The contaminant in weathered contaminated soils is likely to be retained or occluded within small pores, soil aggregates, and soil organic materials. Diffusion of chemicals from these fractions is predicted to be a limiting factor to biotreatment. These soils are likely to have a highly heterogeneous mix of chemicals and metabolites, and microbial communities adapted to this environment (Hatzinger and Alexander 1995).

I incubated four aged contaminated soils (chapter 3), and found that the quantity of C lost from the DEO fraction of two of the four soils was statistically greater than the quantity of C respired as CO_2. The loss of DEO-C was greater than the loss of total soil C in all four soils. An explanation for this discrepancy is that some of the C originally in the DEO fraction was transformed into another soil C fraction, insoluble in dichloromethane or inaccessible to it. Dichloromethane extraction is often used as a measure of hydrocarbon contamination in soils (Lajoie and Strom 1994; Trzesicka-Mylarz and Ward 1995). Another possibility is that C was lost from the soils as volatile organic compounds, however the observation that the total soil C did not decrease significantly supports the hypothesis of C redistribution rather than removal.

Following the transformations of C substrates into one or two soil C fractions requires identification or definition of fractions, and methods to obtain them. Model substrates are usually labeled with ^{14}C, or identified by their $^{13}C/^{12}C$ ratio. Often the soil C fractions are defined in terms of rate of metabolism or ease of extraction, as in "labile C" or "bound C". Added C may be traced into the classical humic component and its humin, humic acid, and fulvic acid constituents (Kohl and Rice 1998; Rüttiman-Johnson and Lamar 1997; Xie *et al.* 1997) . Soil C fractions may be physically based, examining the distribution of substrate C into various clay size fractions (Wander and Traina 1996). Finally, the distribution of added C may be among a wide range of biochemical C fractions. These fractions may be defined mainly by extraction protocol such as 6 M HCl hydrolysis selective for proteins and polysaccharides, dichloromethane extraction (hydrocarbons), and NaOH extraction (humic materials) (Lajoie and Strom 1994).

I tested the hypothesis that during incubation components of DEO are transformed into soil constituents that are insoluble in or inaccessible to dichloromethane during Soxhlet extraction. To do so we monitored the transformation of ^{14}C-labeled model

organic contaminants (MOCs) into six moieties: volatile, CO_2, water soluble, humic, biomass, and DEO. This allowed us to account for redistribution of added C from model contaminants and compare the results to published results on plant residues decomposing in soils.

Materials and Methods

Soils

Two soils were used: an oil-contaminated soil, collected from an agricultural site near Devon Alberta, and a creosote-contaminated soil collected from an industrial site in Edmonton Alberta. The oil-contaminated soil is loam-textured, pH 7.7 (in water) and is contaminated with 5.6 mg DEO g^{-1} soil. The creosote-contaminated soil is silty clay-textured, pH 7.3 (in water), and is contaminated with 1.5 mg DEO g^{-1} soil.

MOC Additions

The ^{14}C-labeling was done on soil incubation units that had been moistened to 50% of their water holding capacity and allowed to equilibrate for 1 wk. The oil-contaminated soil was labeled with [1-^{14}C]octadecane (59.2 Bq g^{-1} soil) and additional non-labeled octadecane was added so that the total concentration of octadecane added was 1.47 mg octadecane g^{-1} soil. The octadecane for each incubation unit (100 g soil) was dissolved in methanol. It was delivered to each soil incubation unit by injecting small volumes of solution into several spots in the soil (>10 points) until all the solution was added. The creosote-contaminated soil was labeled with [4,5,9,10-^{14}C]pyrene (54.9 Bq g^{-1} soil) and additional non-labeled pyrene was added so that the final concentration of pyrene added was 0.29 mg pyrene g^{-1} soil. The pyrene for each incubation unit was dissolved in 0.4 mL toluene. The solution was injected to each soil incubation unit in the same manner as for the oil-contaminated soil. The methanol and toluene were allowed to evaporate from the soils until the solvent odour dissipated (~2 h).

Nutrient Amendment

In all incubations, the soils were supplemented with $(^{15}NH_4)_2SO_4$ (4.5432 % At. Ex), KH_2PO_4, and K_2HPO_4. The oil-contaminated soil received 0.35 mg $(NH_4)_2SO_4$ g^{-1} soil, 0.012 mg KH_2PO_4 g^{-1} soil, and 0.014 mg K_2HPO_4 g^{-1} soil. The creosote-contaminated soil received 0.28 mg $^{15}NH_4SO_4$ g^{-1} soil, 0.010 mg KH_2PO_4 g^{-1} soil, and 0.011 mg K_2HPO_4 g^{-1} soil. The nutrients were dissolved in water and injected into the soil in the same manner as the MOCs.

Incubation

Soil samples of 100 g (oven-dry basis) were weighed into 100-mL glass jars (height 8 cm, diameter 4 cm). Each jar was then placed inside individual 1-L Kerr sealer jars with a vial containing 5 mL 1 M KOH to trap CO_2 and a vial containing 0.5 g of Ambersorb (Sigma) to trap volatile organic C. A moist paper towel was also placed in each Kerr jar to reduce soil desiccation. The soils were weighed biweekly and water added as necessary to keep them at 50% of their WHC as determined by the funnel method (Harding and Ross, 1964) as described in chapter 3. Thus, the creosote-

contaminated soil was kept at 26% gravimetric water content and the oil-contaminated soil at 24%. The jars were sealed and incubated in the dark at 21° ± 1°C. The KOH traps were changed weekly.

The experiment was set up as a completely randomized experiment with the only treatment being incubation time. Each treatment was replicated three times, and destructively sampled at 1 wk, 2 wk, 1 mon and then each month for 11 more mon. Consequently, each soil was added to 42 incubation units at the start of this experiment. In addition, 18 identical, but non-radiolabeled soil incubation units were included for each soil. These were included to allow measurement of total C and ^{15}N quantities in selected samples using equipment not approved for radioactive samples. The non-radiolabeled units were sampled at 1 wk, 1, 3, 6, 9, and 12 mon.

Carbon Fractionation

Figure 4-1 is a flow diagram summarizing the C fractionation process described below.

The KOH traps were collected weekly and 1 mL of each trap was counted by scintillation counting in 9 mL Optiphase "HiSafe" 3 scintillation cocktail to measure the amount of radiolabel evolved as $^{14}CO_2$. The scintillation counter was a Packard Tri-Carb 2000CA Liquid Scintillation Analyzer (Canberra Packard Canada), and the scintillation cocktail used for all the extracts was Optiphase "HiSafe" 3 (Fisher Chemicals, Ltd.).

To account for volatile organic C that may evolve during incubation, the Ambersorb traps were oxidized on a Harvey Biological Oxidizer. The C in samples is oxidized by heating the sample in a stream of oxygen to 935°C. The resultant $^{14}CO_2$ is bubbled into scintillation cocktail made by R.J. Harvey Instruments where it is trapped. The collected sample in cocktail was then counted and the total ^{14}C in the combusted sample determined. To ensure complete and uniform combustion, a few milligrams of non-radiolabeled cellulose is sprinkled over all samples before being placed in the oxidation furnace.

Water soluble-C (WSC) was extracted by the method of Monreal (1983). Subsamples of each treatment (2 g) were shaken with MilliQ water (1:10, w/w) for 1 h on a flat bed shaker (180 cycles min^{-1}), then centrifuged at 2000×*g* for 30 min. The supernatant was collected and ^{14}C content determined by scintillation counting of 1 mL of the supernatant. To collect the humic-C (HC) fraction (Anderson and Schoenau 1993), the soil pellet from centrifugation for WSC was then resuspended in 0.5 M HCl, shaken for 30 min on the flat bed shaker (180 cycles min^{-1}), centrifuged at 2000×*g* for 30 min and the supernatant discarded. The soil pellet was resuspended a second time in 0.5 M NaOH, shaken for 18 h and then centrifuged at 2000×*g* for 30 min. The supernatant was retained for scintillation counting and the pellet discarded. Biomass-C and DEO-C were each determined on fresh, separate subsamples.

To determine biomass-C (BC) by chloroform fumigation-extraction (Joergensen 1995), samples of each treatment were divided into two equal subsamples, "control" and "fumigated". The "control" samples (2 g) were immediately shaken with 20 mL 0.5 M K_2SO_4 for 1 h, then centrifuged at 2000×*g* for 30 min and the supernatant collected for scintillation counting and the soil pellet discarded. The samples to be fumigated were placed in a moist dessicator with a small beaker containing 25 mL of ethanol-free

chloroform. The dessicator was evacuated using a vacuum pump until the chloroform had boiled for 2 min. A "coldfinger" trap containing liquid N_2 was inserted between the dessicator and the pump to prevent chloroform from entering and contaminating the pump oil. The dessicator was then sealed and incubated in the dark 24 h. The next day the beaker of chloroform was removed and the dessicator was evacuated six times for 2 min each, to remove all trace of the chloroform. The fumigated samples (2 g) were then shaken with 20 mL 0.5 M K_2SO_4 for 1 h, centrifuged at 2000×g for 30 min and the supernatant collected for scintillation counting. Biomass was calculated by the following equation:

$$C_{Biomass} = \frac{\left(C_{fumigated} - C_{control}\right)}{k_{ec}} \qquad \text{[Eq. 4-1]}$$

where:

$C_{fumigated}$= the quantity of C (or activity of ^{14}C) measured g^{-1} chloroform-fumigated soil

$C_{control}$= the quantity of C (or activity of ^{14}C) measured g^{-1} non-fumigated control soil

k_{ec}= Biomass C extraction efficiency coefficient (0.25)

Total DEO-C was determined on fresh soil samples of 10-15 g, mixed with an equal amount of dehydrated $MgSO_4$ to ensure dryness. These samples were Soxhlet-extracted overnight with 200 mL CH_2Cl_2. The collected CH_2Cl_2 following Soxhlet extraction was reduced to < 5 mL under vacuum, using a 70°C water bath as a heat source. The reduced extract was then transferred to a 10 mL volumetric flask and brought to 10 mL with fresh CH_2Cl_2. Five millilitres of the extract was transferred to pre-weighed aluminum dishes to measure the tarry residues and the activity of the remainder was counted on the scintillation counter.

The non-radiolabeled samples were subjected to the same C fractionation scheme described above, however the total C contents of the WSC, BC (fumigated), BC (control), and HC fractions were quantified by an Astro 2001 Series 2 Soluble C Analyzer. The system uses an ultraviolet source and 1 M sodium persulphate as the oxidizing agent, 85% phosphoric acid as the sparging solution and was calibrated against an aqueous solution of potassium acid phthalate (100 mg L^{-1}).

Total soil ^{14}C was measured by completely oxidizing 150 mg samples of each treatment in a Harvey Biological Oxidizer. The sum of ^{14}C measured in the WSC, BC, HC, and DEO soil fractions was subtracted from the total soil ^{14}C to yield an estimate of ^{14}C associated with the soil humin (HNC):

$$^{14}C_{humin} = {}^{14}C_{total} - \left({}^{14}C_{WS} + {}^{14}C_{humic} + {}^{14}C_{biomass} + {}^{14}C_{DEO}\right) \qquad \text{[Eq. 4-2]}$$

^{15}N Analysis

Selected non-labeled extracts (20 mL) were Kjeldahl-digested and steam distilled according to the method described by McGill and Figuereido (1993). The kjeldahl digestion procedure entailed reducing the volume of extract to ~10 mL by gently heating (~90°C) in a heating block. The samples were then treated with 10 mL concentrated

sulphuric acid and a kjeltab. The temperature of the block was raised to 220°C and held there for 1.5 h, then raised to 350°C for 3.5 h. After the first 1.5 h (before the temperature was raised) the air condensers were placed on the tubes in the block. The samples were then cooled in the block overnight. To collect the N in boric acid, the contents of the tube were quantitatively transferred to a 500-mL round bottomed flask with 3 rinses of distilled, deionized water. A 100-mL snap-top container containing 5 mL of 2% boric acid solution (containing bromocresol green and methyl red indicator dyes) was placed under the condenser so that the condensor tip was immersed in the acid solution. The flask was attached to the closed distillation apparatus and 25 mL of 25 M NaOH was slowly added as steam was directed into the flask. Approximately 40 mL of distillate was collected. The distillate was titrated with 0.001 M H_2SO_4 until the colour change from green to pink (pH 5.4) was observed. Total N may be calculated by:

$$\mu g\ Total\ N = mL(sample - blank) \times M \times 0.028 \times 10^6 \qquad \text{[Eq. 4-3]}$$

where M is the molarity of the H_2SO_4. The N in the distillate was collected on $KHSO_4$-saturated glass fiber filter paper disks over 6 d in a diffusion procedure in which 5 mL of 25 M NaOH was added to each container to convert all the N in a sample to NH_3 and NO_2 gases. The container was snapped sealed and secured with electrical tape for the duration of the diffusion.

The filter papers were dried overnight in a dessicator and then analyzed on a Carlo Erba NA1500 nitrogen analyzer attached to a VG SIRA (stable isotope ratio analyzer) 10 with a triple collector. The values for total N in the sample (μg) and for percent abundance of ^{15}N are used to determine ^{15}N enrichment in the sample:

$$N_{FS} = \frac{N_S \times {}^{15}N_S}{{}^{15}N_F} \qquad \text{[Eq. 4-4]}$$

where:

N_{FS} = fertilizer-derived N in sample (μg)
N_S = total N detected in sample (μg)
N_F = ^{15}N enrichment of fertilizer applied (percent atmospheric excess)

To correct for mineral N in the sample, non-digested samples of each extract were subjected to the diffusion procedure. Equation 4-4 was applied to this mineral N data and the value subtracted from that obtained above for the organic N.

Statistics

One-way ANOVAs using SAS (Windows 6.10) PROC GLM were applied to the soil C fraction data. A Duncan's multiple range test was used to determine significant differences within each soil C fraction ($p<0.05$). As well, a non-parametric one-way procedure was applied to compare the distribution of ^{14}C remaining in the soil among all treatments. The non-linear procedure described in chapter 3 was used to fit the $^{14}CO_2$ production curves to three kinetic models (Gompertz, logistic growth, or exponential growth). The coefficient of multiple determination, R^2 was also calculated as described in chapter 3.

Results

Oxidation of the Ambersorb traps from both soils for the first 6 months yielded no significant amount of ^{14}C, indicating no significant loss of volatile organics. Based on these results and the almost 100% recovery of applied ^{14}C in the last 6 months, the remaining Ambersorb traps were not analyzed.

The total C measurements in the WSC, BC, and HC fractions of both soils varied little during the incubation (Fig. 4-2). The HC peaked on day 91, the same day on which the ^{14}C enrichment of the HC peaked as well (Fig. 4-3). The total C in the HC fraction did not decrease significantly after this date. The total C content of the HC fraction on day 91 was also the peak for the whole incubation, however this value does not correspond with the HC-^{14}C value.

The ^{14}C-pyrene was quickly mineralized in the creosote-contaminated soil, such that 50% of the applied label was lost as $^{14}CO_2$ within the first 3 months (Fig. 4-4). The rate of CO_2 production peaked sharply in the fourth week of incubation and decreased steadily to an approximately constant rate by 6 mon. At the end of the incubation, 69% of the applied label had been evolved as $^{14}CO_2$. The production of the CO_2 from both soils best fit the exponential model (Table 4-1). The oil-contaminated soil also fit the Gompertz model, however the residual sum of squares for the 3-parameter Gompertz model was greater than that for the 2-parameter exponential model. Therefore, the exponential model was accepted. The implications of the exponential model being the best fit are that substrate availability is the only limiting component to metabolism and that there are ample microorganisms to mediate the reaction. This also implies that there is a very small lag time before the microorganisms respond to added substrate by mineralizing it. The exponential model may be represented as:

$$C_{CO_2} = C_0\left(1 - e^{-kt}\right) \qquad \text{[Eq. 4-5]}$$

where:

C_0 = the potentially mineralizable C (Bq)
k = a rate constant (d^{-1})
t = time (d)

The DEO-^{14}C (Fig. 4-5) in the creosote-contaminated soil decreased significantly during the first 63 d of the incubation. There was no further significant change in DEO-^{14}C over the remaining 300 d, as it maintained a mean value of about 5.8% of the applied ^{14}C. The HC-^{14}C, BC-^{14}C, and WSC-^{14}C (Figs. 4-6 and 4-7) fractions fluctuated slightly during the 12 months, however their ^{14}C activities remained minor (<8% of the applied ^{14}C in each fraction) for the duration of the incubation.

The ^{14}C-octadecane applied to the oil contaminated soil was metabolized quickly as well; 47% of the applied label was lost as $^{14}CO_2$ within 3 mon (Fig. 4-4). After 12 mon of incubation, 68% of the applied label had been evolved as $^{14}CO_2$. Similarly to the creosote-contaminated soil, the kinetics of C mineralization in the oil-contaminated soil are best described by the exponential model (Table 4-1), indicating again that the microorganisms are able to quickly begin mineralizing the added substrate.

As with the creosote-contaminated soil samples, the DEO-^{14}C (Fig. 4-5) of the oil-contaminated soil decreased significantly during the first 63 d of the incubation, and then leveled off at 17% of the applied ^{14}C. For the subsequent 300 d of the incubation, DEO-

^{14}C did not change significantly. The BC-^{14}C and WSC-^{14}C (Fig. 4-6) also fluctuated slightly for the duration of the experiment, but never exceeded a combined total of 12% enrichment from the applied ^{14}C.

The behaviour of the MOC in the oil-contaminated soil differed from that in the creosote-contaminated soil, in that the HC (Fig. 4-3) was a site of significant ^{14}C enrichment in the oil-contaminated soil. The ^{14}C activity in the HC increased significantly and substantially to 32% of the total applied ^{14}C in the second month of the incubation. The activity remained the same for the next 4 mon, then decreased slightly until the last month when the value was not significantly different from the first months.

For the oil-contaminated soil, the biological oxidizer measurements of total soil ^{14}C were variable, so that the overall changes are rarely significant. The mean value for HNC-^{14}C (oil contaminated soil) was 40% of the total ^{14}C applied. The HNC values for the creosote-contaminated soil are presented in Fig. 4-6.

The creosote-contaminated soil had specific activity values ranging from 0.001 to 0.01 Bq μg^{-1} C for both WSC and BC. The oil-contaminated soil had specific activity values ranging from 0.003 to 0.01 Bq μg^{-1} C for both WSC and BC. The ^{14}C:^{12}C in the HC fraction of the oil-contaminated soil is highest at 0.005 Bq μg^{-1} C at 91 d, the sampling date on which the ^{14}C enrichment of the HC peaked as well. Conversely, the ^{14}C:^{12}C in the HC fraction of the creosote-contaminated soil is extremely small (4.6×10^{-5} Bq μg^{-1} C) at 91 d, and the total C content of the fraction is the maximum for the whole incubation.

The gravimetric DEO content of the creosote-contaminated soil decreased significantly over the incubation, from 1.57 mg DEO g^{-1} soil to 0.67 mg DEO g^{-1} soil (Fig. 4-8). The gravimetric DEO content of the oil-contaminated soil did not change during the incubation (Fig. 4-9), maintaining a mean value of 5.1 mg DEO g^{-1} soil.

Total C recoveries for both soils are displayed in Fig. 4-10.

Non-labeled BC extracts and HC extracts were analyzed for ^{15}N enrichment for three dates: 2 wk, 3 mon, and 12 mon. There is evidence of fertilizer-derived N incorporation into BC at days 14 and 91 and into HC at days 14, 91 and 364 (Table 4-2).

Discussion

I must begin by addressing the final recovery of the applied ^{14}C. The initial experimental protocol did not include a measurement of Humin (HN). Because the early observations indicated that I was not accounting for 100% of the applied ^{14}C in the creosote-contaminated soil, it was proposed that the remainder might be in humin. The measurement of HN was intended to ensure a 100% accounting of the applied radiolabel. Recovery of the ^{14}C applied as octadecane to the oil-contaminated soil was greater than 100%, ranging between 116% and 143%. This suggests that some of the C fractions overlap with one another, leading to C_{total} accountings that are erroneously high. The recovery of ^{14}C from pyrene in the creosote-contaminated soil also averages near 100% for the last 6 mon of the incubation. However, the first 6 months of incubation for this system have very low recoveries, ranging from 62-81 %. While undesirable, poor recoveries are not unusual when working with radiolabeled contaminants in soil (Lajoie and Strom 1994; Rüttiman-Johnson and Lamar 1997). Since no volatile ^{14}C was detected during the first 5 months of the incubation, and 100% recovery was achieved in the latter part of the incubation, it appears that volatilization was not the cause of the poor

recovery. The increased total ^{14}C recovery measured from 6 mon to the end of the incubation would be due to an increasing uniformity of substrate distribution.

Both soils contained dichloromethane-extractable tarry residues as a component of their contamination, so it had been predicted that the MOCs would partition into the non-aqueous phase liquid (NAPL) and be inaccessible to microorganisms (Efroymsen and Alexander 1995). The log K_{OW} of pyrene is 5.32 (Sims and Overcash 1983). The longest chain alkane on lists of chemical constants that include log K_{OW} or the constants needed to calculate K_{OW} is dodecane (C_{12}), with a log K_{OW} reported to be approximately 6 (MacKay *et al.* 1993). As it is known that log K_{OW} increases with increasing chain length, the log K_{OW} for octadecane will be >6. Within 12 wk of incubation, both soils had respired almost 50% of the applied ^{14}C. The rapid mineralization suggests either that the predicted partitioning did not occur, or that the indigenous microorganisms were capable of mineralizing the substrate in the NAPL. It is possible that the endogenous NAPL is not uniformly distributed through the soil and may not be in close proximity to the injected MOC, hence the partitioning could not occur. This may be due, in part, to residual NAPLs remaining protected within stable aggregates. The capability of the microorganisms with respect to metabolizing substrates in a NAPL phase remains a question to be tested.

Contrary to what was observed in chapter 3, the DEO content of the oil-contaminated soil did not change during this incubation. As was discussed in the Research section of chapter 1, the soils for these two experiments came from different sampling occasions. It is possible that the lower initial DEO content of this sample (compared to the ESTAC stocks) is a threshold to further removal of the residues.

Interestingly, Dechênes *et al.* (1996) and Lajoie and Strom (1994) both found mineralization patterns that are similar to those described here for radiolabeled pyrene in soil. Dechênes *et al.* (1996) found that >50% of the applied pyrene had disappeared within the first 12 wk of incubation in a weathered creosote-contaminated soil. Lajoie and Strom (1994) added radiolabeled pyrene to a soil artificially contaminated with coal tar oil and inoculated with an adapted microbial culture and found that >50% of the applied ^{14}C was respired as $^{14}CO_2$ within the first 12 wk, which included a 4-wk lag phase at the beginning of the incubation.

In spite of the artificial system, the work of Lajoie and Strom (1994) poses an interesting comparison with mine, as they too, found very little ^{14}C enrichment in the BC (<5%) and HC (7%) fractions after 12 wk of incubation.

In my incubation, it might also have been expected that there would be a relationship between C evolved as CO_2 and WSC, however none was found. This is not evidence that the microorganisms are metabolizing the substrate from a non-aqueous form, though it is possible. It may be that the substrate is rapidly moving through the aqueous phase in low concentration as part of the dynamics of metabolism.

The BC values may be combined with the cumulative $^{14}CO_2$ data to calculate ^{14}C utilization efficiencies (^{14}CUE) for each month (Eq. 4-7, Fig. 4-10).

$$CUE = \frac{BC}{(BC + C_{Cum CO_2})} \qquad \text{[Eq. 4-7]}$$

The ^{14}CUE values for the first 2 wk of incubation of the creosote-contaminated soil are extremely high, as the amount of $^{14}CO_2$ respired was very small relative to the quantity of

^{14}C in the BC fraction. For the next 11 months of incubation, the ^{14}CUE values remain much lower, ranging between 0 and 6%. This is a normal pattern for CUE to follow as the incubation progresses over time. A similar interpretation could be made for octadecane metabolism in the oil-contaminated soil based on the calculations of ^{14}CUE. The ^{14}CUE values for the oil-contaminated soil are highest at week 1 (15.1%), but never again exceed 11% for the rest of the incubation. The detection of fertilizer-derived N in the biomass as well as in the HC fraction is further evidence that the C redistribution was through biologically-mediated transformations, rather than abiotic processes.

It is also of interest that the ^{14}C content of the HC fraction in the oil-contaminated soil reached a peak of 32% at day 91 and then decreased for the next 9 mon, eventually reaching the HC content of week 1. This raises questions about contaminant attenuation: Is the process a dynamic humification, or has the contaminant or its metabolites diffused into this fraction and are slowly being released? Since the specific activity of ^{14}C in the HC of the oil-contaminated soil is higher than in any other C fraction of either soil (except the original DEO-C fraction), I assume that this was the most dynamic fraction. Given that the DEO-^{14}C was decreasing while HC-^{14}C was reaching a peak, it is possible that the MOC-^{14}C was transformed into the HC fraction, rather than sorbed into it. However, the observation that the HC-^{14}C ultimately drops down to the level of week 1 indicates that the modification that facilitated the MOC association with HC was such that the modified MOC remained primarily in a labile HC pool. Alternatively, if the MOC was sorbed into the HC fraction, and subsequently released, one should be able to identify controls on these two reactions. Such controls are not readily apparent. Alternatively, it is possible that the MOC was sorbed abiotically into the HC fraction, and subsequently released. The observation of fertilizer-derived ^{15}N in the HC fraction, with the ^{14}C enrichment suggests, though does not prove, that biotransformation may be the mechanism.

Studies of contaminant-C distribution in soil have found substantial enrichment of the HN fraction with this C (Murphy *et al.* 1990; Xie *et al.* 1997). Humin is an operational component of soil humus defined as non-extractable in water at any pH (Aiken *et al.* 1985). My calculations of HN ^{14}C for each soil were based on variable measurements, so few differences between months for each soil are significant. A direct measurement would be the most accurate means of quantifying HN, but my values do suggest that transformation of contaminant-C into HN-C is certainly a process involved in bioremediation.

This experiment clearly showed the great potential for redistribution of contaminant hydrocarbons into soil organic C fraction. Combined values ranging between 10 and 74% of the applied ^{14}C was estimated to be enriching the HC and HNC fractions in both soils during the experiment. Though the enrichment of HC fraction in the oil-contaminated soil reached a peak of 32%, it appears that this peak is transient, while the enrichment of the humin, though only calculated by difference, may be much more stable, in both soils. This is consistent with the convention that humin is slower to turn over than are humic substances. Establishing how transient or stable these enrichments may be is important not only to bioremediation technologies, but also to sustainable soil practices.

It is also clear from the $^{14}CO_2$ respiration data that the microbial biomass is an important mediator of these reactions, however the total ^{14}C enrichment of the BC fraction does not immediately reflect this. An examination of the carbon utilization and

incorporation kinetics would yield important information about the unique characteristics of contaminant hydrocarbons and their degrading microorganisms. This kind of information could help scientists refine the conditions considered optimal for C sequestration and bioremediation.

The main conclusions reached from this experiment are: 1) freshly added contaminants may not interact with or be sorbed by residual "aged" organic contaminants in contaminated soils; 2) redistribution of contaminant-derived C appears to be through biologically-mediated transformations; 3) transformations of contaminant-derived C into stable humin and humic-C components of SOM can be substantial and may reach as high as 75% of the added substrate; 4) these transformations may be transient. Further studies should examine the nature of the aged residues in soil in order to design more effective remediation systems. As well, stabilizing the humification or incorporation into humin of contaminant residues is a desirable fate of contaminants and should be a goal of bioremediation research.

Table 4-1. Parameters for the exponential model, describing the kinetics of $^{14}CO_2$ production in both soils.

Soil	C_0 (Bq g^{-1} soil)	k (d^{-1})	R^2
Creosote-contaminated	38.3 ± 11.1	0.013 ± 0.0054	0.55
Oil-contaminated	34.2 ± 5.02	0.021 ± 0.0044	0.85

Table 4-2. ^{15}N concentration in BC and HC at 2 weeks, 3 months, and 12 months (from the non-radiolabeled samples).

Fraction	Time	µg fertilizer-derived N g^{-1} soil (std. dev.)
Biomass-C	2 wk	1.00 (0.28)
	3 mon	0.60 (0.27)
	12 mon	0
Humic-C	2 wk	4.27 (0.55)
	3 mon	2.64 (0.93)
	12 mon	3.47 (0.64)

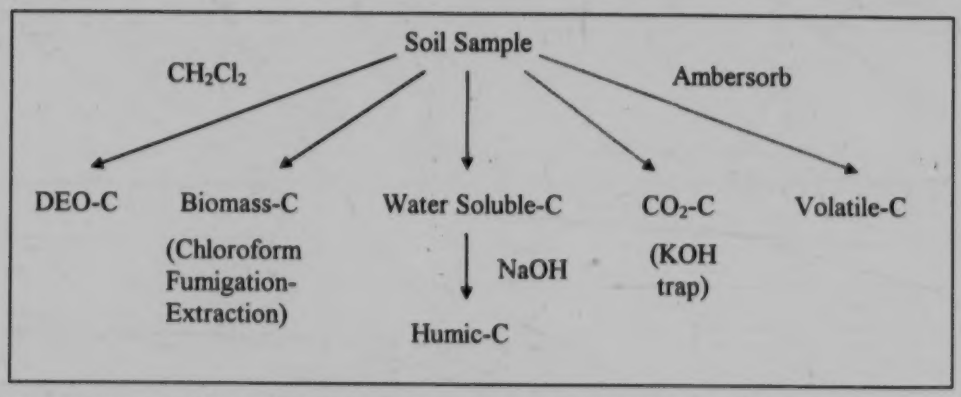

Figure 4-1. Soil C fractionation scheme. Each solid line from "Soil Sample" represents a distinct subsample. The dashed lines represent traps included in the incubation apparatus.

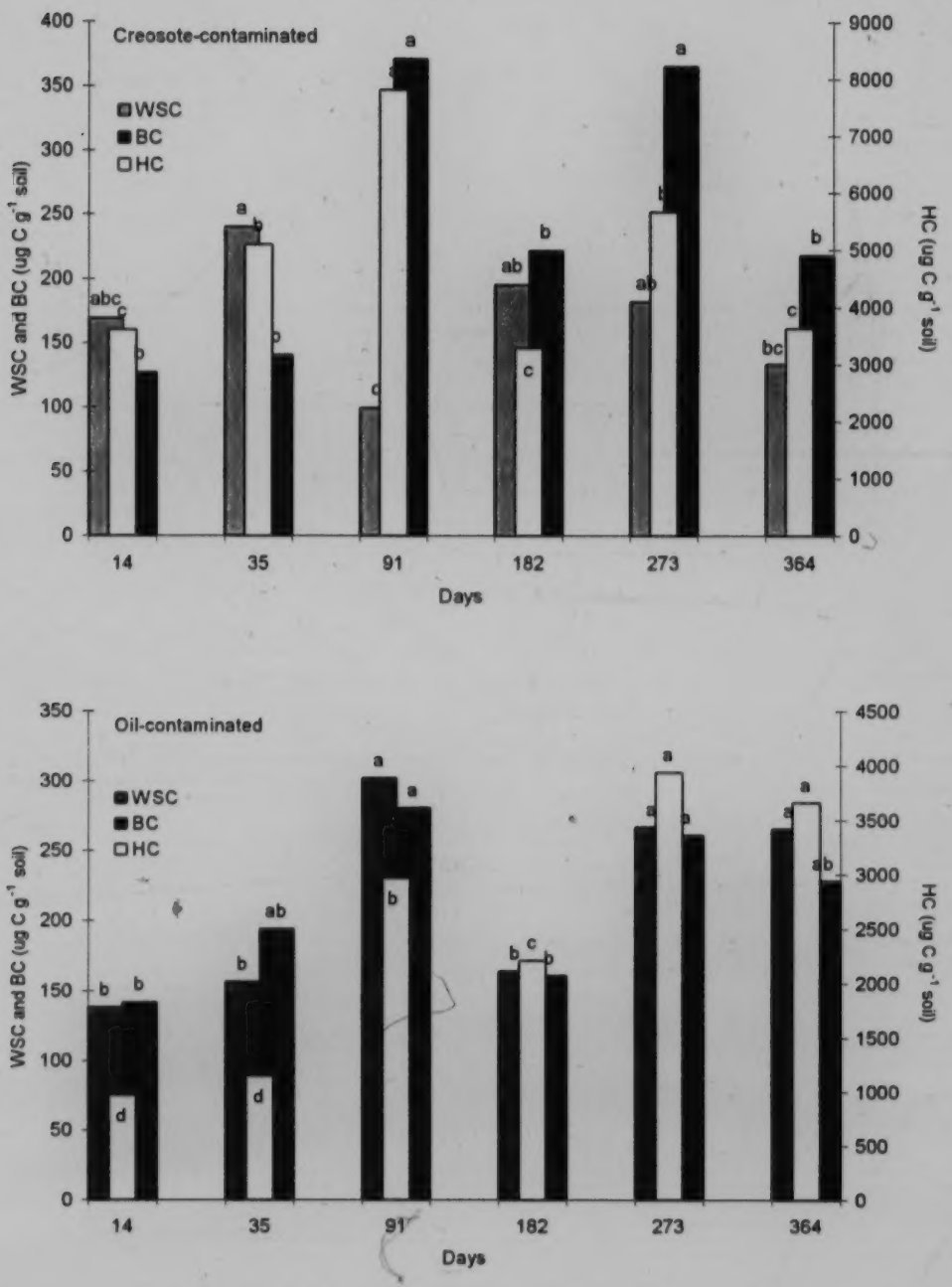

Figure 4-2. Concentration of C in both soils; data collected from the non-labeled incubation units. Bars topped by the same letter are not significantly different ($p<0.05$); comparisons are within fractions, among days.

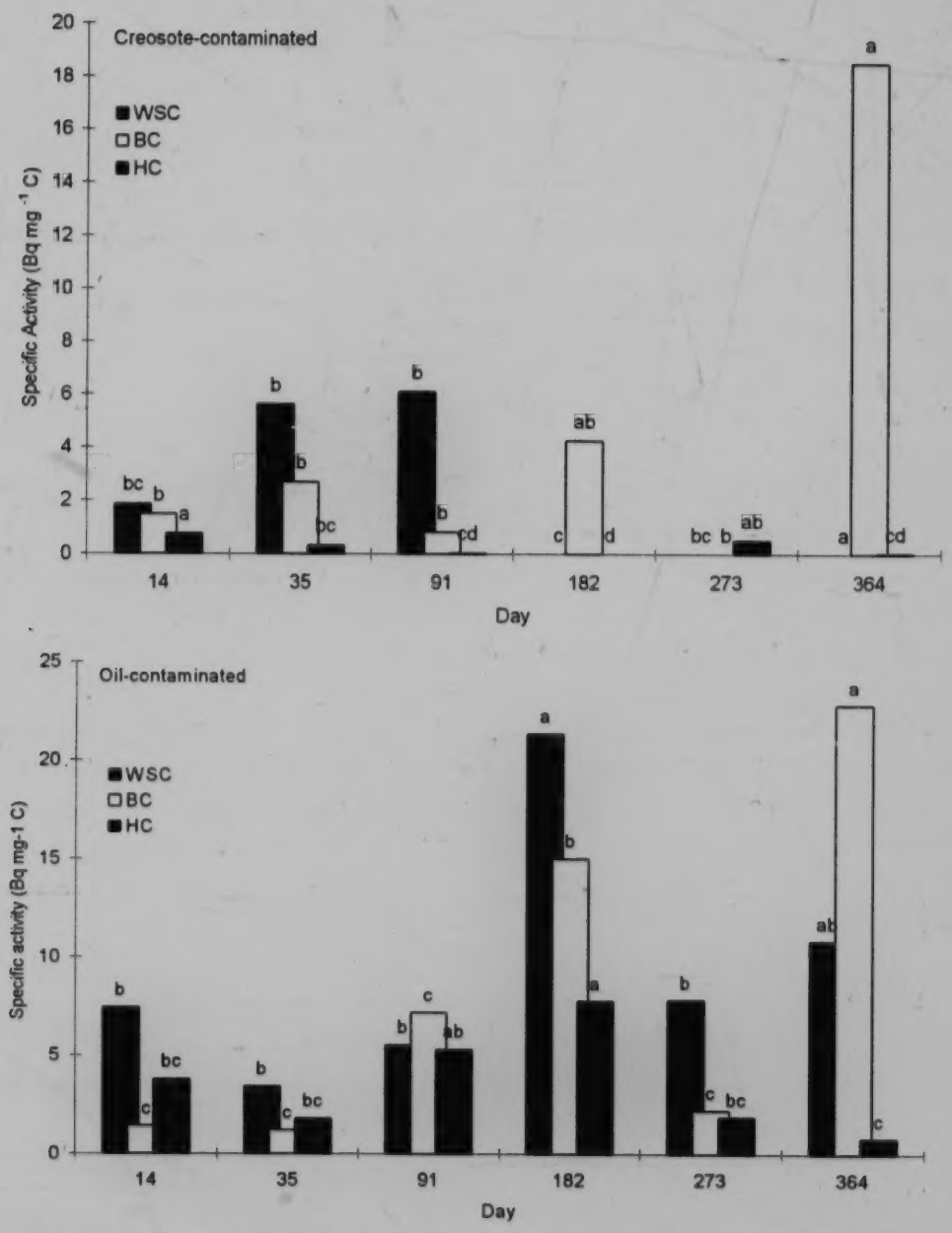

Figure 4-3. Specific activities of three C fractions of two contaminated soils spiked with MOCs, measured at six sampling dates over 12 mon. Statistical comparisons made within fractions, among days. Bars topped by the same letter, within fractions, are not statistically different ($p<0.05$).

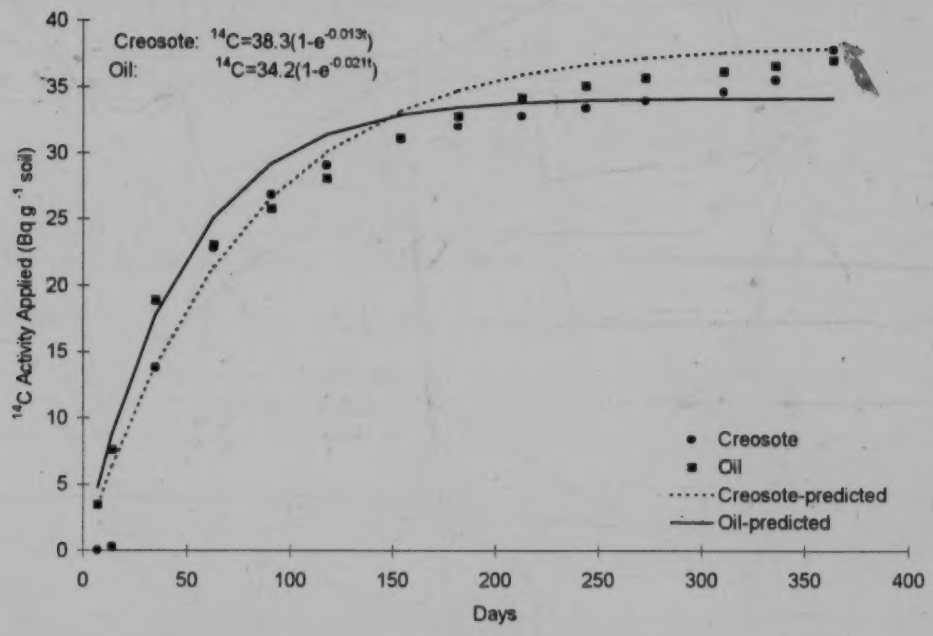

Figure 4-4. Cumulative CO_2-^{14}C evolution from a creosote-contaminated soil spiked with [4,5,9,10-^{14}C]pyrene (initial activity 54.9 Bq g^{-1} soil) and an oil-contaminated soil spiked with [n-^{14}C]octadecane (initial activity 59.2 Bq g^{-1} soil), incubated for 12 mon.

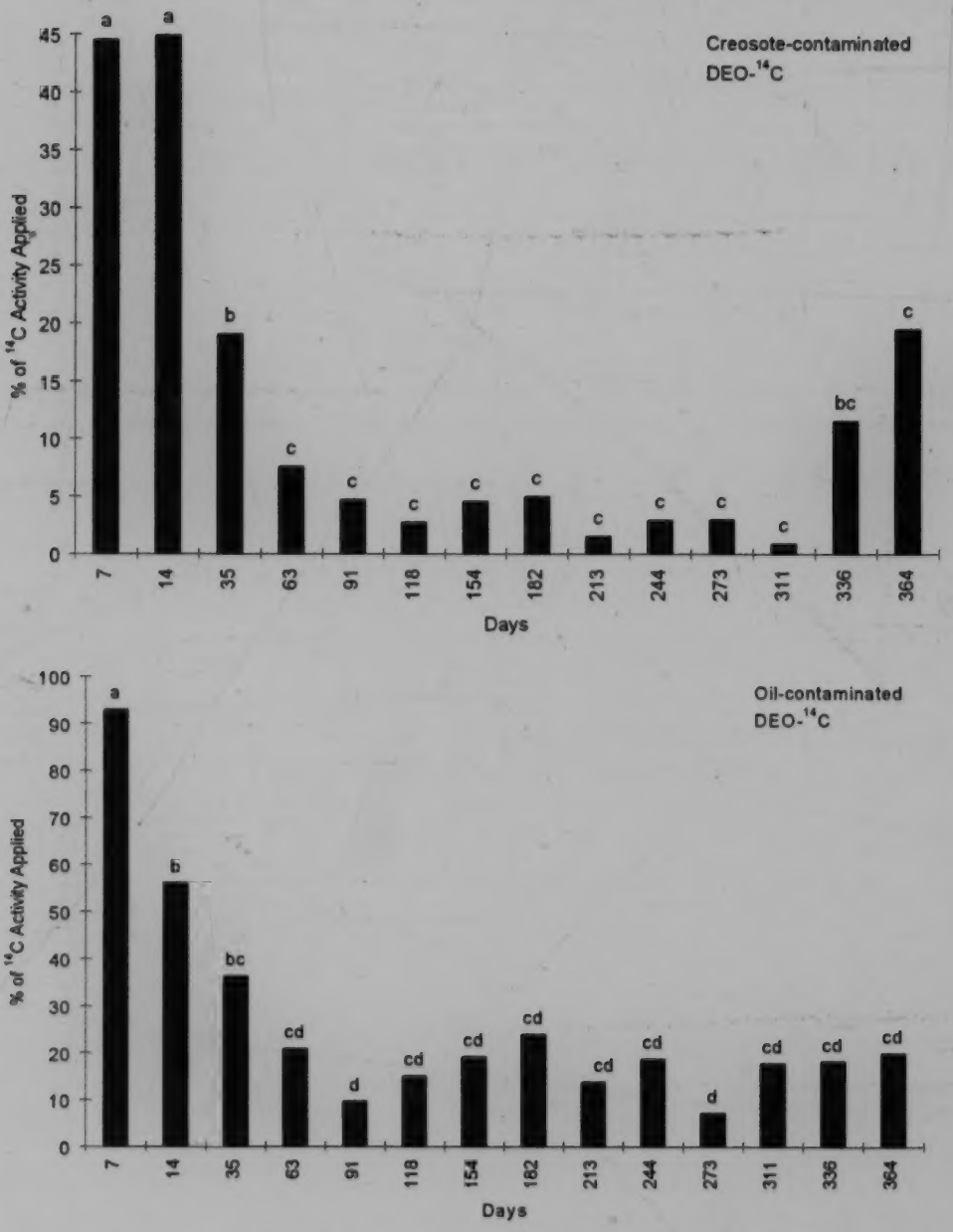
Creosote-contaminated
DEO-14C
% of 14C Activity Applied
Days
a a b c c c c c c c c bc c
7 14 35 63 91 118 154 182 213 244 273 311 336 364
Oil-contaminated
DEO-14C
% of 14C Activity Applied
Days
a b bc cd d cd cd cd cd cd d cd cd cd
7 14 35 63 91 118 154 182 213 244 273 311 336 364

Figure 4-5. ^{14}C recovery in the DEO fraction of a creosote-contaminated soil spiked with [4,5,9,10-^{14}C]pyrene and an oil-contaminated soil spiked with [n-^{14}C]octadecane, incubated for 12 months. Bars topped by the same letter are not significantly different ($p<0.05$).

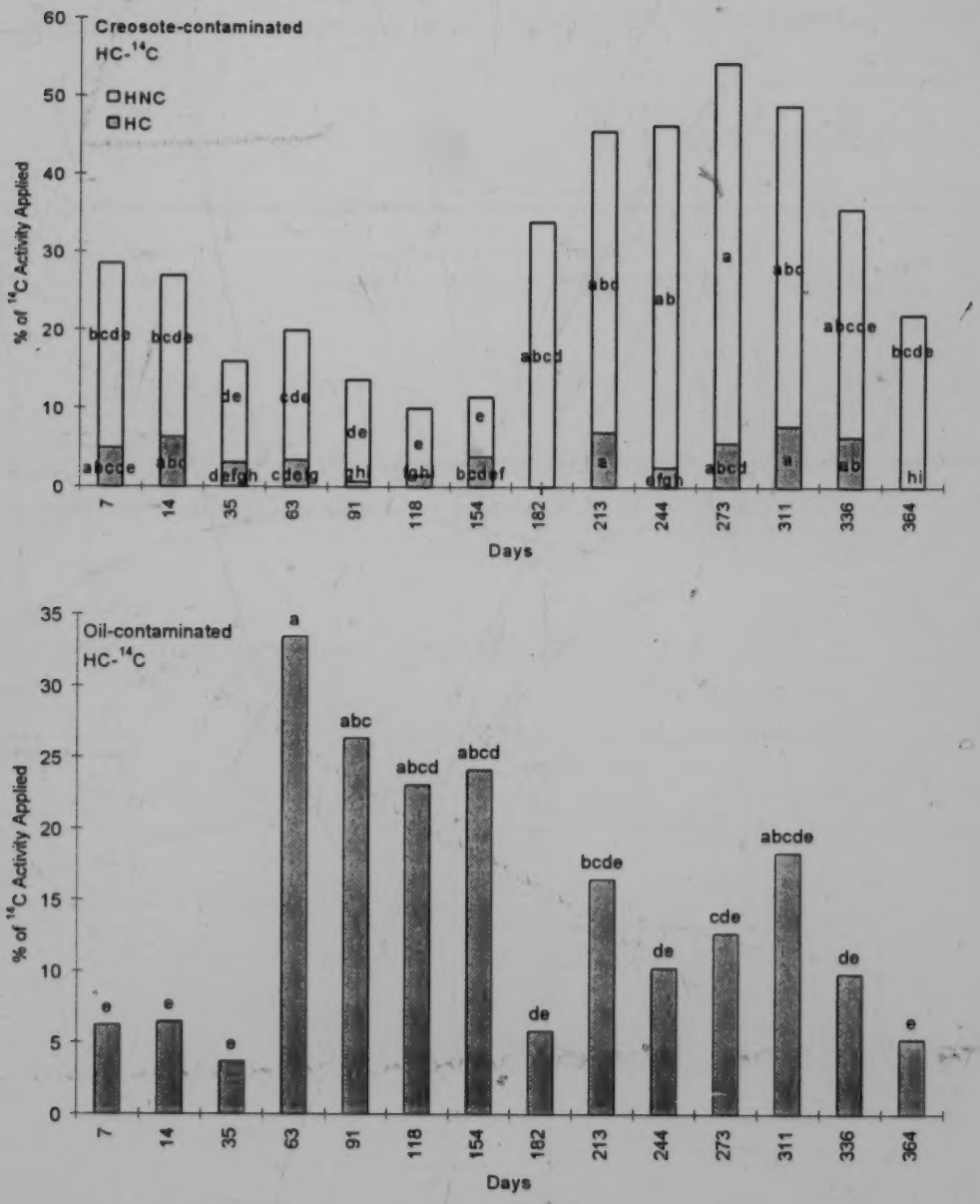

Figure 4-6. ^{14}C recovery in the HC and HNC fractions of a creosote-contaminated soil spiked with [4,5,9,10-^{14}C]pyrene and an oil-contaminated soil spiked with [*n*-^{14}C]octadecane, incubated for 12 mon. The grey bar represents the entirety of the HC; the whole of the white bar (does not include grey component) is HNC. No significant differences were detected in HNC for the oil-contaminated soil; mean HNC=40%. Bars containing the same letter are not significantly different ($p<0.05$); comparisons only made within fraction, among months.

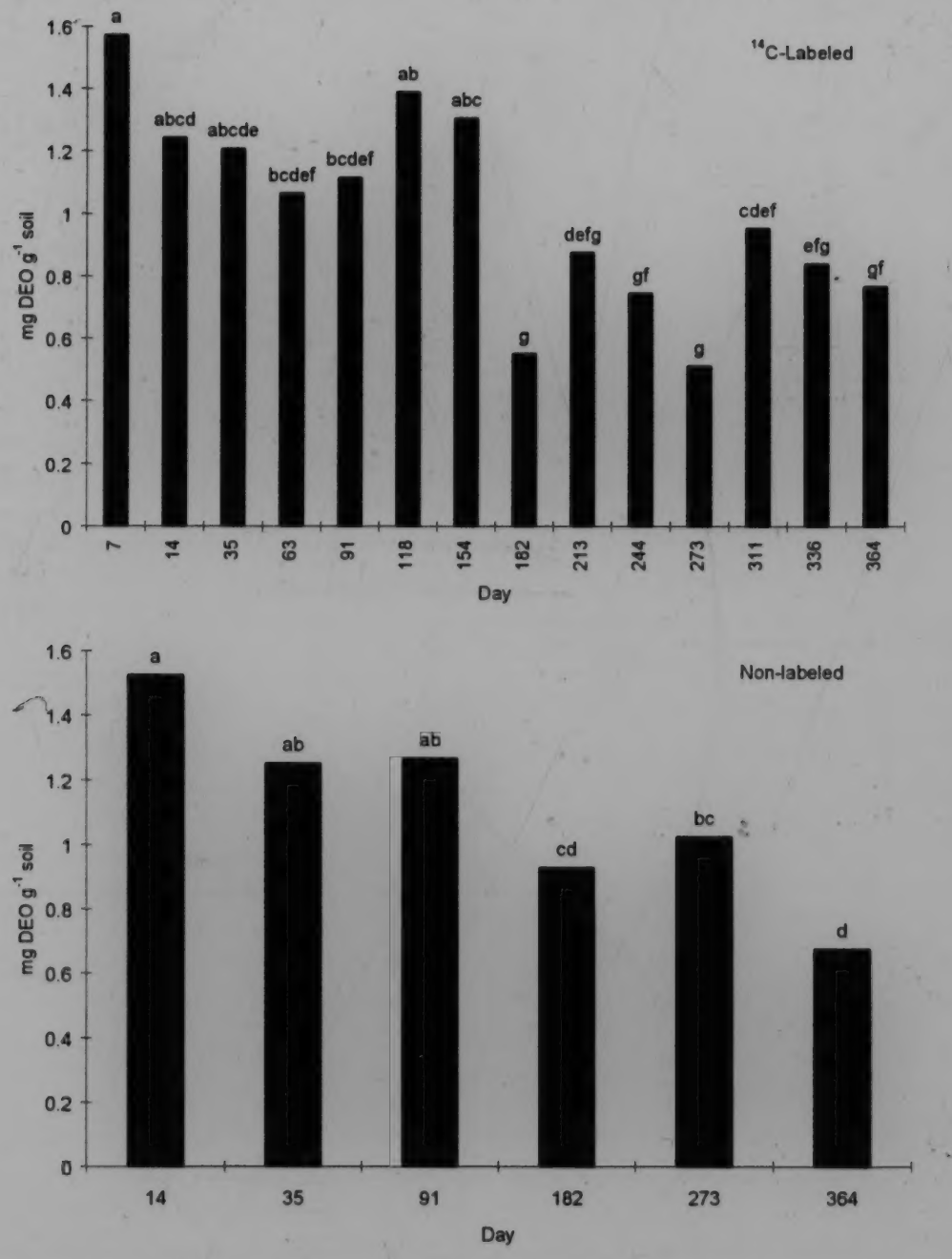

Figure 4-7. Gravimetric DEO contents of the creosote-contaminated soil during the incubation; both ^{14}C-labeled and non-labeled samples shown. Bars topped by same letter are not significantly different.

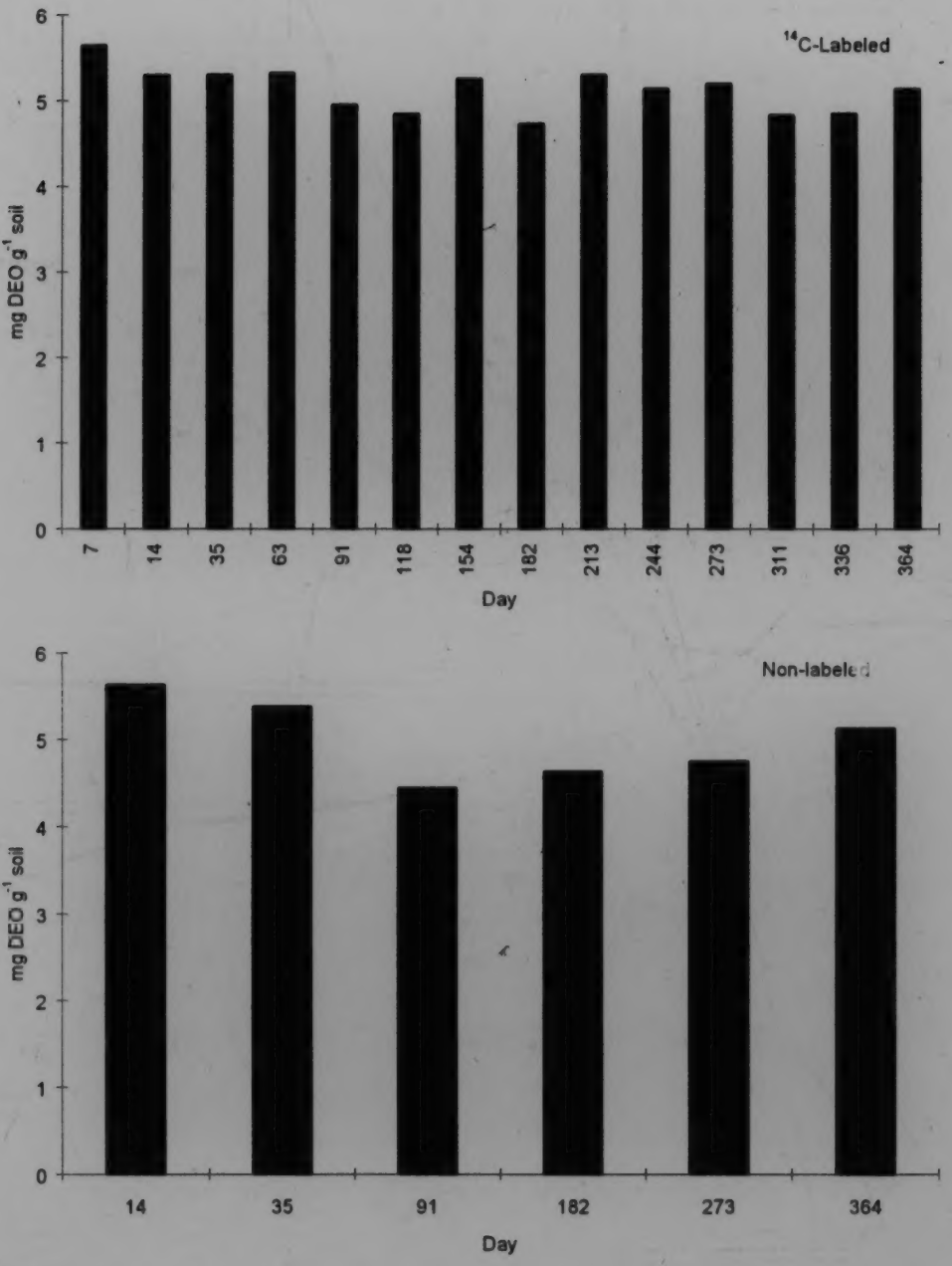

Figure 4-8. Gravimetric DEO contents of the oil-contaminated soil during the incubation; both ^{14}C-labeled and non-labeled samples shown. No significant changes were detected.

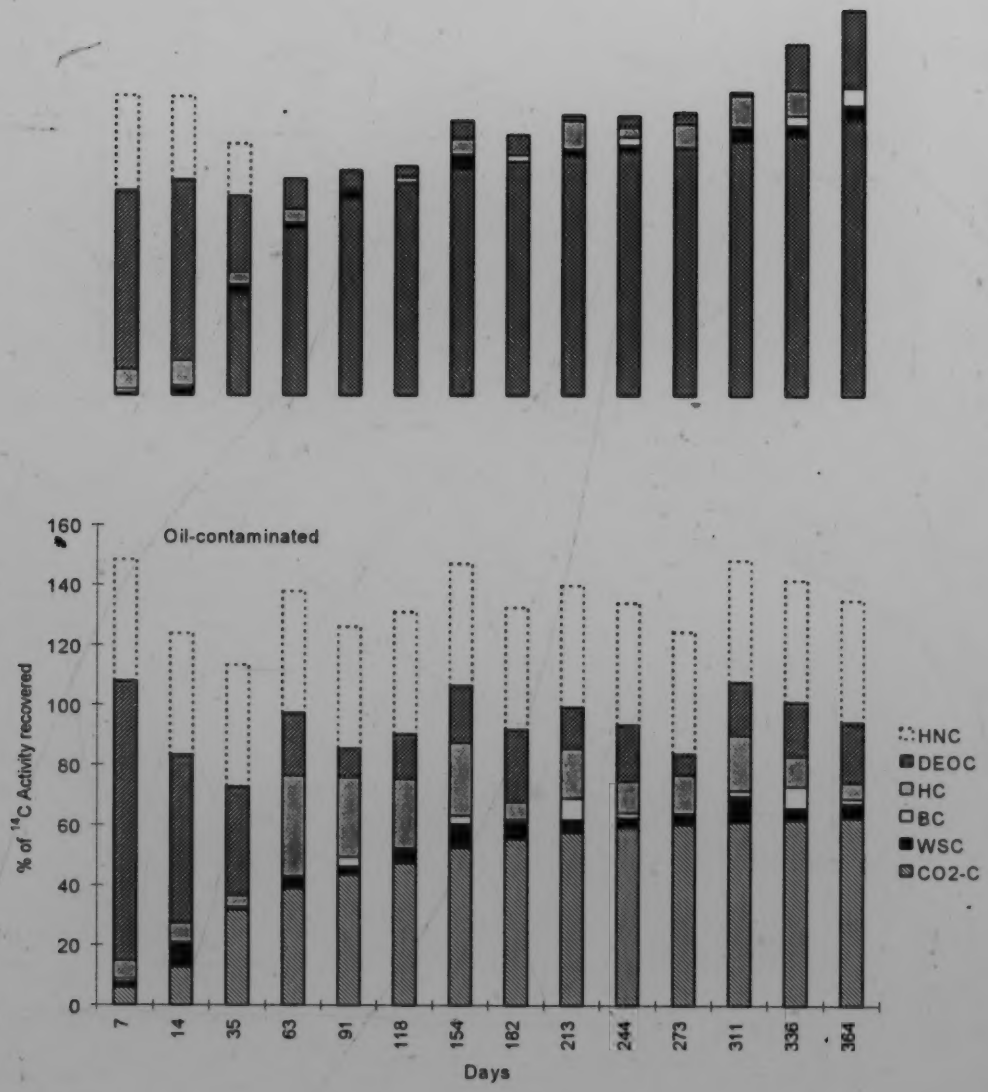

Figure 4-9. Stacked bars show recovery of added ^{14}C during the incubation.

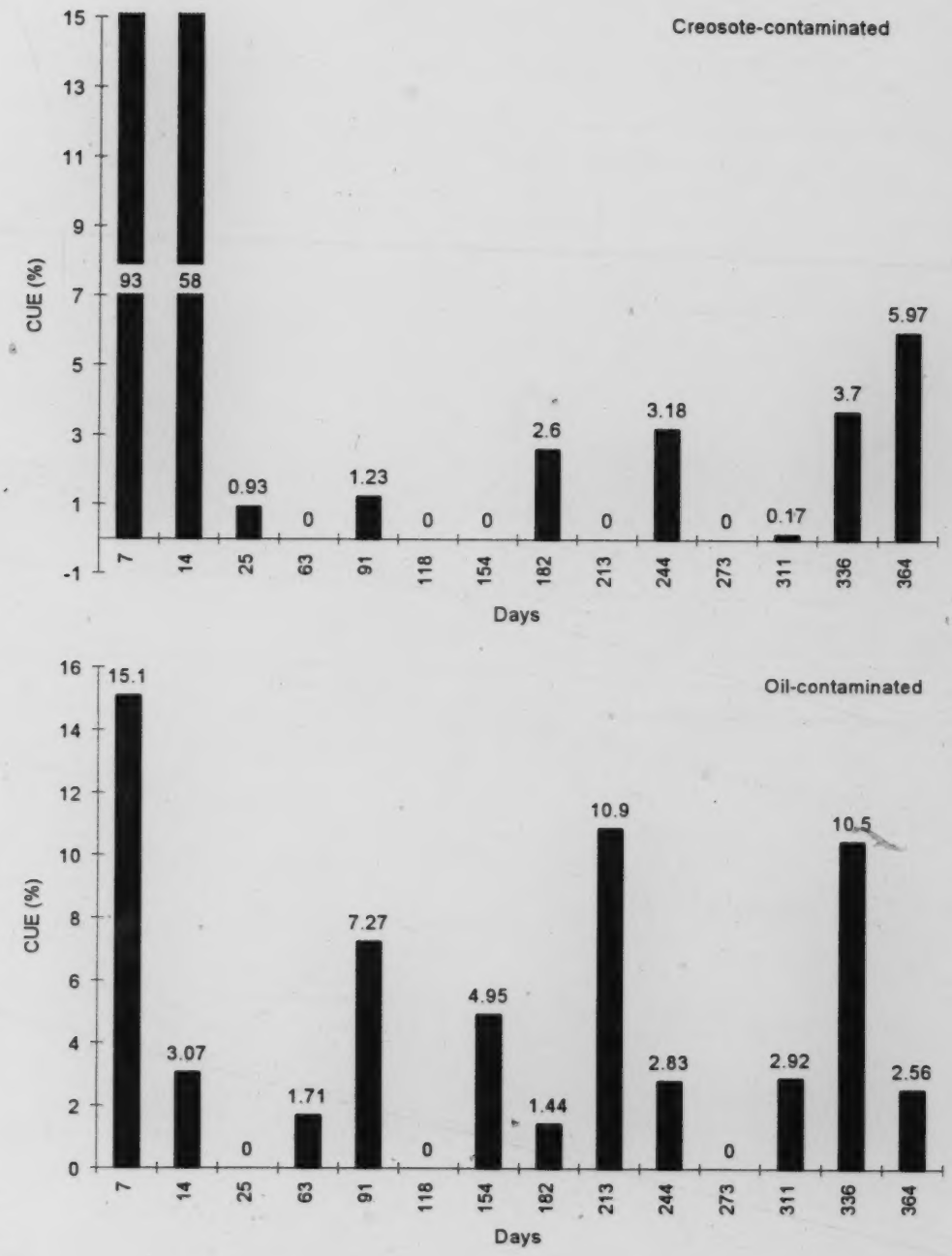

Figure 4-10. Calculated ^{14}C utilization efficiencies (^{14}CUE) for a creosote-contaminated soil spiked with ^{14}C-pyrene and an oil-contaminated soil spiked with ^{14}C-octadecane.

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CHAPTER 5

THE PRESENCE OF NON-AQUEOUS PHASE LIQUIDS IN WEATHERED CONTAMINATED SOILS

Introduction

Bioremediation is a biological method for removing contaminants from soils. The end result of successful bioremediation of hydrocarbons is the conversion of contaminant-C to carbon dioxide (CO_2), biomass, or the transformation of the contaminant into less harmful or less mobile metabolites (Wilson and Jones 1993).

Often the contaminants, as is the case with oil and creosote, in sufficient quantities, include non-aqueous phase liquids (NAPLs) (Peters and Luthy 1993). NAPLs may be water-immiscible hydrocarbons that can change the way organic contaminants behave in soil (Efroymson and Alexander 1994; Fu and Alexander 1995), often due to the K_{ow} of the compound. The K_{ow} is a measure of the affinity a compound has for an organic phase, relative to an aqueous phase. Specific organic contaminants may partition into NAPLs to such a degree that their concentration in the aqueous phase falls below the threshold for biodegradation (Efroymson and Alexander 1994). The resulting reduction in bioavailability is often a major constraint to the removal of a compound from soil (Atlas and Cerniglia 1995; Pollard *et al.* 1994).

The NAPL itself may be quantified in soil by extracting the soil with an organic solvent, such as dichloromethane. A challenge to describing weathered contaminants in soils is determining whether solvent-extractable organic materials neccessarily indicate the presence of a NAPL. It is possible that the non-aqueous solvent-extractable phase may exist as a film coating soil materials; distinguishing this from a completely sorbed phase may be difficult.

The weathering of contaminants in soils results in a different medium than does artificial or fresh contamination of soils. Weathered contaminated soils may have the contaminant distributed through the soil in an uneven fashion. Contaminant constituents located at microbe-air-water interfaces, such as the surface of aggregates and mouths of larger pores are the most readily metabolized (Rosenberg *et al.* 1992). The constituents located within stabilized aggregates or intimately associated with the soil mineral and organic matrix is much less bioavailable, and therefore, more persistent (Hatzinger and Alexander 1995).

Hydrophobic compounds sorb preferentially to hydrophobic components of soil organic matter (Walton *et al.* 1989). It has been observed that the behaviour of these compounds is similar in the presence of a NAPL, and that the NAPL may sorb up to 10 times more compound than the soil organic matter (SOM; Boyd and Sun 1990). The result of partitioning into a NAPL is reduced bioavailability.

I report here on two experiments, parts of a series of studies dealing with C transformations in soils contaminated and weathered with oil or creosote. Two soils have been subjected to a series of experiments to examine the processes of bioremediation: an oil-contaminated soil and a creosote-contaminated soil.

I reported previously on a 12-mon incubation of two weathered contaminated soils (chapter 4). One of these soils had a loam texture and was contaminated with oil; the other was silty clay textured and was contaminated by creosote. These two soils were spiked with radiolabeled model contaminants. The oil-contaminated soil samples were spiked with [1-^{14}C]octadecane and the creosote-contaminated soil samples were spiked with [4,5,9,10-^{14}C]pyrene.

After 10 wk, 60% of the radiolabel added to the creosote-contaminated soil samples and 50% of the radiolabel added to the oil-contaminated soil samples had been recovered as $^{14}CO_2$. With respect to the creosote-contaminated soil samples, the pyrene was metabolized to a much greater extent than would be predicted by its log K_{ow}, 5.32 (Sims and Overcash 1983), seemingly not having partitioned into the NAPL phase present in the soil. It was also noted that 35% of the radiolabel added to the oil-contaminated soil samples was recovered in the humic C fraction.

Questions arose from these results, primarily: Did radiolabeled model contaminants partition into the aged NAPL in these two soils? Is the high mineralization rate due to a lack of partitioning? Yeom *et al.* (1996) noted that a rate-limiting step in the bioremediation of PAHs in soils containing a NAPL was the release of PAH from the original NAPL. I examined these questions by adding extra NAPL from additional samples of the same soil, together with ^{14}C-octadecane or ^{14}C-pyrene that were added separately or mixed into the NAPL. I expected the quantity of $^{14}CO_2$ evolved from the ^{14}C-pyrene samples to decrease; and the recovery of ^{14}C from octadecane in the humic C fraction in the oil-contaminated samples to decrease as the model contaminants partition into the fresh NAPL. I refer in this paper to the DEO (dichloromethane-extractable) materials as existing in the soil as NAPLs; this may not be so, as previously discussed. I make this generalization based on the strong petroleum odour of both soils that indicates the presence of a vapour phase and on the visibility of an oily phase in the soil when sampled. It is possible that these materials sorbed to soil constituents during the storage of the soils prior to use.

The work reported here had two objectives: 1) to determine if improving the accessibility of the NAPL to the MOC will increase the degree of partitioning, and hence, decrease the oxidation or transformation of the MOCs; and 2) to determine if there is a critical amount of NAPL that must be present in the soil for this partitioning to occur.

Methods and Materials

Soils

The two soils have previously been described in chapter 4.

Experimental Design

Objective 1: The availability of NAPL required for partitioning of the MOC into extra NAPL was examined through two treatments for each soil. The MOCs were added to the soils in two ways: 1) injected into several points in the soil sample 3 h after the NAPL was added ("NAPL+^{14}C") or, 2) mixed with the NAPL and then added to the soil ("(NAPL+^{14}C)"). For both treatments, the quantity of NAPL added was twice that

present in the soil (measured as DEO) and it was added in 5 mL of dichloromethane. The KOH traps included in the apparatus were collected and counted every 3 days.

Objective 2: Only the creosote-contaminated soil was studied. The quantities of NAPL added to the incubation units were varied: 1×, 0.75×, 0.5×, 0.25×, and 0× the amount of NAPL added to the creosote-contaminated soil incubation units in experiment one. All of these proportions of NAPL were added in 5 mL dichloromethane, including the 0× treatment which had 5 mL pure dichloromethane added. Following this treatment, the soils were spiked with the ^{14}C-pyrene, as described for objective 1. The KOH traps were collected and counted weekly.

The experimental design for both objectives was completely randomized design with three replicates. The treatments for objective were "NAPL+^{14}C" and "(NAPL+^{14}C)", applied to two soils for a total of 12 experimental units. The treatments for objective 2 were the five rates of NAPL amendment applied to the creosote-contaminated soil for a total of 15 experimental units.

MOC Additions

Objective 1: the oil-contaminated soil was spiked with [1-^{14}C]octadecane (45 Bq g^{-1} soil) and the creosote-contaminated soil with [4,5,9,10-^{14}C]pyrene (45 Bq g^{-1} soil).

Objective 2: the creosote-contaminated soil was spiked with [4,5,9,10-^{14}C]pyrene (66 Bq g^{-1} soil).

NAPLs

Both objectives: the soils were enriched with NAPL which had been Soxhlet-extracted overnight with dichloromethane from reserve stocks of each soil.

Nutrient Amendment

Both objectives: the soils were supplemented with $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4 as described in Chapter 4.

Incubation

Small jars (100 mL) containing 50 g (oven dry basis, ODB) of each soil were placed inside individual 1-L Kerr sealer jars with a vial of 5 mL 1 M KOH to trap CO_2. A moist paper towel was also placed in each Kerr jar. Maintenance of the incubation followed the procedure described in chapter 4. The KOH traps were changed and scintillation-counted every 2 d for objective 1 and weekly for objective 2.

Each treatment was replicated three times and the duration of both incubations was 10 wk.

Carbon Fractionation

The extraction procedures followed were the same as those described in chapter 4, however the Ambersorb traps were not included (Fig. 5-1).

Statistics

One-way ANOVAs using SAS (Windows 6.10) PROC GLM were applied to the soil C fractions and the cumulative CO_2 quantities evolved by the soils at the end of the

incubations. A least significant difference was used to determine significant differences within these data ($p<0.05$). A non-parametric one-way procedure (PROC NPAR1WAY) was used to compare the C distributions among treatments. A χ^2 test was used to determine differences. A separate nonparametric 1-way procedure was used to compare the lines defining the CO_2 evolution rates among treatments within a soil. A χ^2 test was used to compare the sum of scores for each line and determine if they were different ($p<0.05$). The $^{14}CO_2$ production rate data from objective 1 was fit to the kinetic models, as described in chapters 3 and 4. There were too few data points for objective 2 to model the data with confidence.

Results

In objective 1, at the end of 10 wk, only 0.6% of the ^{14}C added to the creosote-contaminated soil samples was recovered as $^{14}CO_2$ (Fig. 5-2), one-one hundredth of the amount of ^{14}C recovered when the same soil is incubated with the model contaminant in the absence of additional NAPL (chapter 4). The amount of ^{14}C recovered as $^{14}CO_2$ from the oil-contaminated soil samples was 28%, approximately half the amount recovered in the second incubation (Fig. 5-2). The amount of ^{14}C recovered in the HC fraction of the oil-contaminated soil samples was 5% of the total, one-seventh of the amount recovered in the second incubation (Fig. 5-3).

The $^{14}CO_2$ production curves for the oil-contaminated soils (objective 1) best modeled by the Gompertz model (Table 5-1). This suggests that under the conditions of this experiment, the C mineralization system began with a lag between addition of substrate and substrate mineralization. The mineralization kinetics were also subject to a decay in mineralization efficiency. The exponential model converged with the creosote-contaminated soil CO_2 evolution, however the calculated R^2 values were negative and greater than 1. The model does not describe our data.

The distributions of C at the end of the objective 1 experiment did not differ with method of ^{14}C addition for both soils, indicating that the method of MOC-NAPL addition was not a factor in the reduction of MOC accessibility to microbes.

The CO_2 production curves for all treatments of objective 2 (Fig. 5-4) do not differ significantly. As well, the distribution of C among each of the respired C, WSC, HC, BC, DEO-C, and HNC fractions follow no clear trends with treatment (Fig. 5-5).

Discussion

The manner in which the model contaminants and NAPLs were added to the soil did not significantly change the redistribution of the carbon in either soil at the end of 10 wk (Fig. 5-3). The rates of $^{14}CO_2$ production, and total $^{14}CO_2$ produced were not significantly different between treatments for the creosote-contaminated soil samples (Fig. 5-2). The total CO_2-^{14}C did not differ greatly between the two treatments of the oil-contaminated soil; however the kinetics of mineralization of ^{14}C from the (NAPL+^{14}C) treatment was observed to have a greater lag time than the NAPL+^{14}C treatment.

The results of objective 1 show that freshly applied NAPL reduces the metabolism of pyrene and octadecane in soils. This is supported by literature that has reported that oils in soils act as "highly efficient partition media" for organic contaminants (Boyd and Sun 1990). Residual polychlorinated biphenyl (PCB) oils were measured to be 67-times

more effective than OM, and 3.5× more effective than octanol, as a sorbent for 2-chlorobiphenyl (Sun and Boyd 1991). However, it was noted that the sorption curves suggested that there may be a threshold concentration of PCB-oil required for it to function as a separate partitioning phase. Further work from the same laboratory indicated that the only organisms that were able to access sorbed naphthalene as substrate were a *Pseudomonas putida* strain that was able to enhance desorption. The organism without that ability was not able to metabolize the naphthalene (Guerin and Boyd 1997).

Considering this observation along with the observations of high rates of MOC oxidation reported for the same two soils without the additional NAPL in chapter 4 leads to a conclusion that it is microbial inaccessibility to, rather than inability to metabolize, that accounts for the persistence of pyrene and octadecane in soils containing a NAPL. Since the experiment reported in chapter 4 resulted in more extensive $^{14}CO_2$ production than did either soil when spiked with model organic contaminant in the presence of additional NAPL, I may also conclude that newly added compounds appear not to partition into the original NAPL.

It was wondered if there was a threshold level of additional NAPL below which, the model organic contaminant would not partition. It was also wondered if the additional NAPL was a factor in the previously observed partitioning, or if the dichloromethane in which it was added was the key factor in redistributing the NAPL already present. Mott *et al.* (1990) suggested that an impediment to bioremediation may be the occlusion of the target contaminant within soil aggregates, therefore the addition of pure dichloromethane may be sufficient to redistribute the protected NAPL such that the MOC would still partition into it.

Experiment 2 answered the second question posed above, and in doing so acknowledged that the first question was not answerable by this experimental design: the application of pure dichloromethane (0× NAPL) to Edmonton soil resulted in a soil C fractionation pattern similar to that of the 1× treatment. From this I may make two inferences: 1) the solvent alone redistributed the endogenous NAPL through the soil to locations closer to the injected contaminants and; 2) the apparent ability of the solvent to redistribute these materials suggests that they are present as a NAPL, rather than as a completely sorbed soil phase. The additional NAPL caused no further reduction in pyrene mineralization, beyond that measured in the presence of solvent alone. This observation rendered the question of NAPL thresholds to partitioning moot. It is possible that the solvent killed the soil microorganisms, however as will be shown in chapter 6, the metabolism of glucose and cellulose was not modified by the addition of extra NAPL added in dichloromethane.

These results indicate that NAPLs in contaminated soils that have weathered over decades are no longer uniformly distributed through the soil. Physical, chemical, and biological weathering processes have resulted in the diminishment of the NAPL at the aggregate surfaces, and that which remains is occluded within the aggregates or is highly recalcitrant to further metabolism. Thus, I can respond to objective 1, by concluding that by increasing the proximity of the MOC to a NAPL, whether by adding fresh NAPL or by redistributing NAPL already present, the degree of partitioning of the MOC into the NAPL is increased. I cannot respond to objective 2, as I have already observed that the design of these two objectives, which used dichloromethane to add the extra NAPL to the

soil created an artifact (the redistribution of endogenous NAPL) that did not allow resolution of this objective.

Table 5-1. Gompertz model parameters describing the kinetics of $^{14}CO_2$ production in the oil-contaminated soil (objective 1).

Treatment	Model Parameters			
	C_0 (Bq g^{-1} soil)	K (d^{-1})	h	R^2
NAPL + ^{14}C	12.64 ± 3.54	0.056 ± 0.025	0.087 ± 0.11	0.78
(NAPL + ^{14}C)	10.66 ± 0.93	0.168 ± 0.010	0.000108 ± 0.00010	0.97

Fumigation-
Extraction)

Humic C

Figure 5-1. Soil C fractionation scheme. Each path from "Soil Sample" represents a distinct subsample. The dashed lines represents a trap included in the incubation apparatus (modified from Chapter 4).

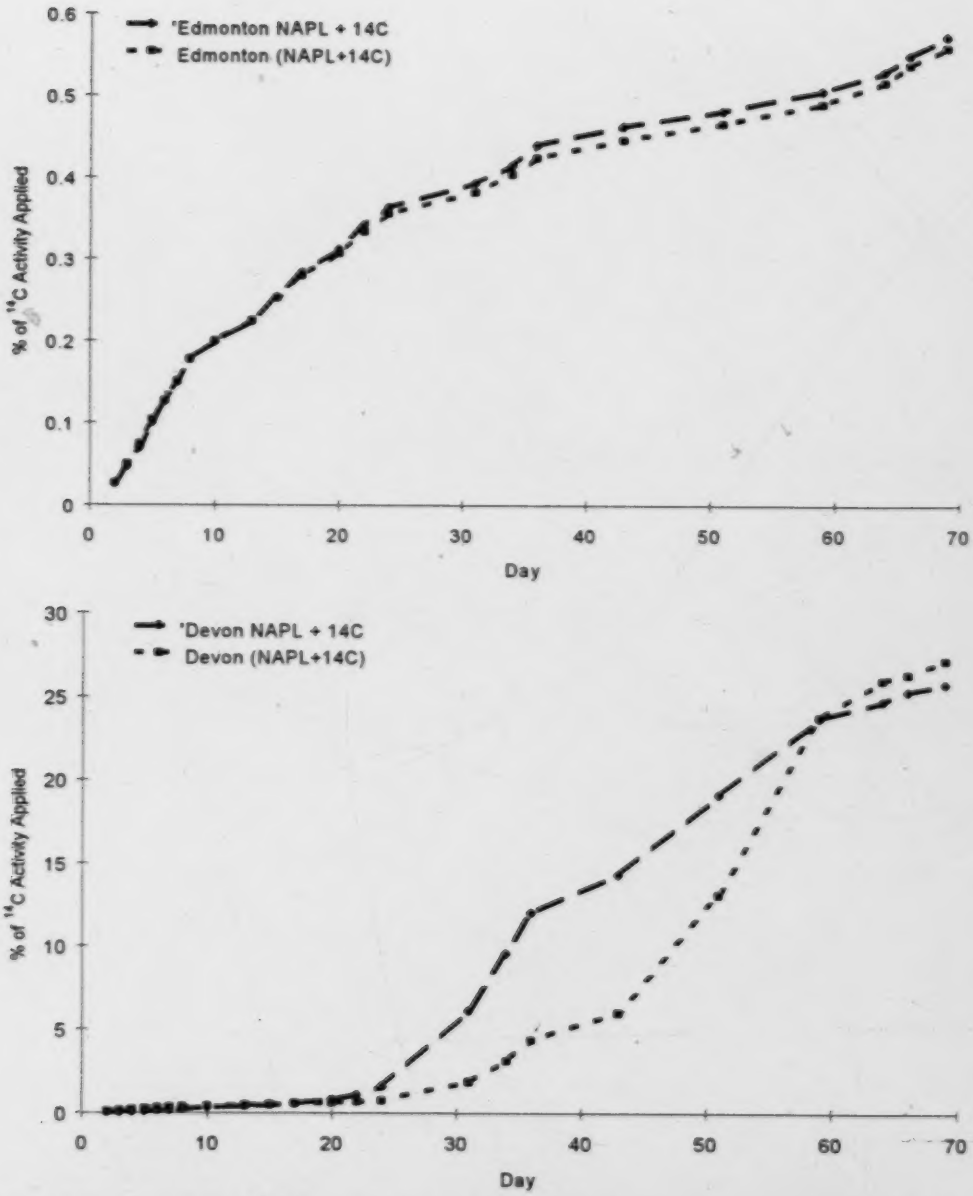

Figure 5-2. Cumulative $^{14}CO_2$ production over 10 weeks from creosote-contaminated soil samples spiked with ([4,5,9,10-^{14}C]pyrene) or oil-contaminated soil samples spiked with ([1-^{14}C]octadecane), by two methods of ^{14}C-model contaminant addition. CO_2 production curves are significantly different for the oil-contaminated soil (χ^2 probability >0.999), not for the creosote-contaminated soil. Total CO_2 evolved at the end of 10 wk not significantly different ($p<0.05$).

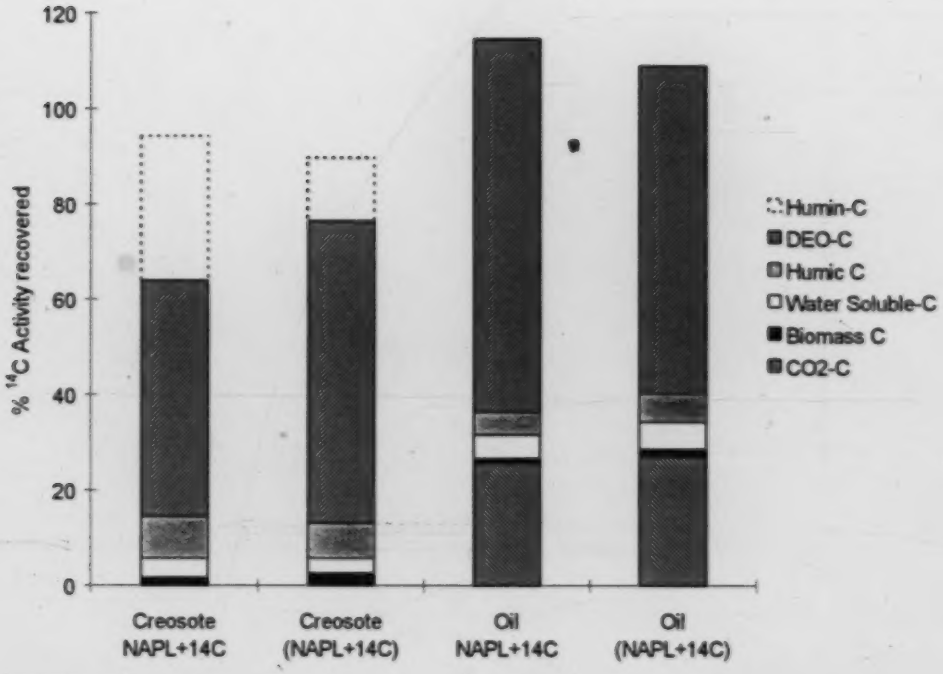

Figure 5-3. Redistribution of ^{14}C from [4,5,9,10-^{14}C]pyrene spiked in a creosote-contaminated soil and from [1-^{14}C]octadecane spiked in an oil-contaminated soil after 10 wk of incubation. Percent ^{14}C activity within soil C fractions are not significantly different within soils. The distribution of C in a soil does not differ significantly between treatments ($p<0.05$).

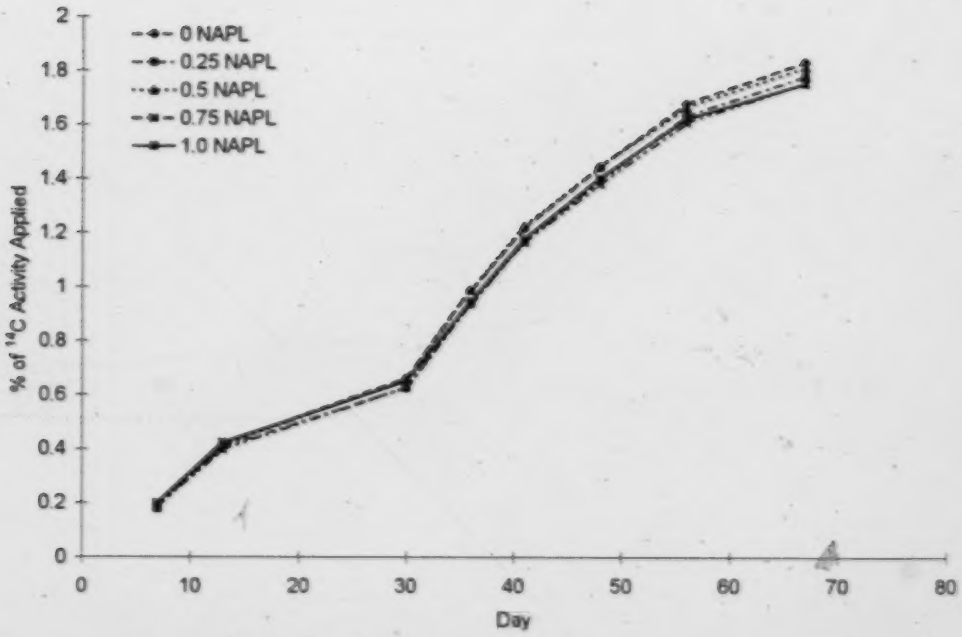

Figure 5-4. Cumulative $^{14}CO_2$ production over 10 wk from a creosote contaminated soil spiked with ^{14}C-pyrene in the presence of: 1×, 0.75×, 0.5×, 0.25×, and 0× the amount of additional NAPL added in experiment 1. The curves do not differ significantly based on χ^2 comparisons (χ^2 probabilities for all comparisons >0.999).

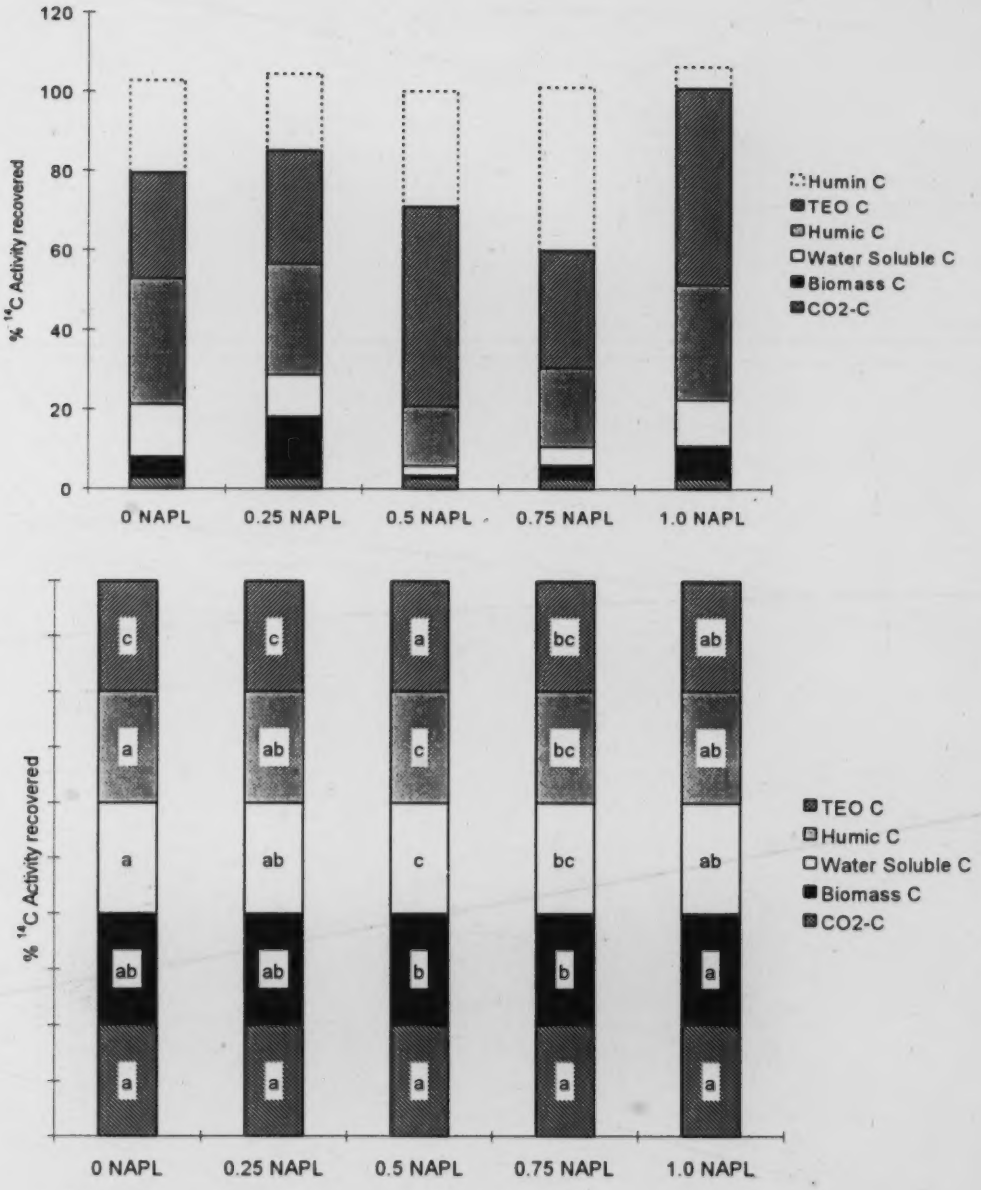

Figure 5-5. Redistribution of ^{14}C from [4,5,9,10-^{14}C]pyrene spiked in a creosote-contaminated soil in the presence of 0×, 0.25×, 0.5×, 0.75×, and 1× the amount of NAPL added to the creosote-contaminated soil in experiment 1. Incubation was for 10 wk. Statistical differences are within C fractions among NAPL rates; comparisons not made among fractions. There were no significant differences in total ^{14}C from which HN-C was determined ($p<0.05$).

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CHAPTER 6

FATE OF EXOTIC C IN TWO WEATHERED CONTAMINATED SOILS

Introduction

In considering the fate of contaminant-derived C in soils, it is useful to use more extensively studied compounds as reference substrates, against which the target contaminant transformations may be compared.

Many microorganisms are observed to preferentially use specific types of substrates in soil, yielding information about the individual organism or suite of organisms, or about the substrate itself. For example: a suite of soil bacteria, studied for their denitrifying capabilities were supplied with acetate, propionate, butyrate, glucose, or sucrose as a C substrate (Paul *et al.* 1989). During the first 24 h of incubation, the microorganisms preferentially metabolized the carbohydrates. In experiments such as these with whole soils, it is highly possible that the organisms being targeted for their unique capabilities are not the same organisms mineralizing the added substrates. One must also be cautious of reported "priming effects" of substrates such as glucose in which the stimulation of the microbial biomass by the readily available substrate enhances the mineralization of native soil organic matter (SOM) (Shen and Bartha 1997). The use of radiolabeled substrates will protect against this overestimation of mineralization.

Other substrates that are studied in soil systems are those that are often found in soil. Cellulose is widely studied as a substrate because it is a component of plant tissues. The results of one experiment examining the decomposition of cellulose in soil found that biomass-C derived from pure cellulose peaked after 40 d, whereas the biomass-C peaked at 80 d when the cellulose was obtained from plant tissue (Scheu *et al.* 1993). In another study of C degradation of forest soils, it was found that the presence of cellulose facilitates the concurrent degradation of lignin, a more complex substrate (Entry and Backman 1995), though this is not commonly considered a "priming effect".

Chotte et al. (1998) examined the fate of several different radiolabeled substrates in soil, with respect to their assimilation into microbial biomass and their overall turnover. After 66 d of incubation in soil, they found that 63% of the applied glucose had been mineralized to CO_2. The contrasting substrates were also metabolized to a similar degree: starch (64%), legume tissues (59%), and wheat tissues (51%). Differences in the transformations of substrate derived-C did show up, as starch amendment resulted in the greatest quantity of substrate-derived biomass (610 $\mu g\ C\ g^{-1}$ soil), followed by glucose (550 $\mu g\ C\ g^{-1}$ soil), legume (350 $\mu g\ C\ g^{-1}$ soil), and wheat (290 $\mu g\ C\ g^{-1}$ soil). Apparently, while the degree of C mineralization may be similar for all four substrates, their microbially mediated fates are not. The association of each substrate with density-separated organic matter (OM) fractions also varied through the experiment

The metabolic potential within two weathered contaminated soils has been reported in this thesis in an effort to characterize contaminated soils that have aged for several decades without treatment. Having examined the fate of a model organic contaminant (MOC) in each of these soils, another question arose: How capable are the hydrocarbon-adapted organisms in these soils of metabolizing non-hydrocarbon

substrates? As a final phase of this series of experiments, different exotic C substrates were applied to each of these soils. The alternate substrates used were radiolabeled glucose and cellulose in both soils. The MOC used in the previous studies of the oil-contaminated soil (chapter 4, chapter 5) was a straight-chain aliphatic hydrocarbon (octadecane), and the primary pathway of degradation for such compounds is beta-oxidation resulting in the loss of C_2 units. Consequently, acetate was also included for the oil-contaminated soil as an analogue for the degradative process. In addition, when MOCs were previously applied to these soils, pyrene, a polycyclic aromatic hydrocarbon, was applied to the creosote-contaminated soil. In this final study, the model organic contaminants were "crossed": the PAH applied to the oil-contaminated soil, and the aliphatic hydrocarbon applied to the creosote-contaminated soil. This was to ascertain whether the previously observed patterns of metabolism for these compounds were due to metabolic processes characteristic of the substrate, to metabolic processes characteristic of the degrading organisms in each soil, or were a combination of the two. If the latter was the case, it would be expected that the CO_2 production patterns, and final C distributions after 10 wk of incubation would be unique, and unlike the patterns seen for either the soil or the substrate in the earlier studies.

Materials and Methods

Soils

The two soils have been described previously in chapter 4.

MOC Additions

Incubation units of the oil-contaminated soil (50 g oven-dry basis) were labeled with one of four C substrates: [4,5,9,10-^{14}C]pyrene (26.7 Bq g^{-1} soil), [U-^{14}C]glucose (186 Bq g^{-1} soil), [U-^{14}C]cellulose (94.5 Bq g^{-1} soil), and [U-^{14}C]acetate (31.8 Bq g^{-1} soil). The creosote-contaminated soil units were labeled with one of three substrates: [1-^{14}C]octadecane (26.6 Bq g^{-1} soil), [U-^{14}C]glucose (186 Bq g^{-1} soil) and [U-^{14}C]cellulose (94.5 Bq g^{-1} soil). The octadecane added to incubation units was dissolved in 0.5 mL acetone and the pyrene added to incubation units was dissolved in 0.5 mL toluene. Both glucose and acetate added to (separate) incubation units were dissolved in 1 mL aqueous solution. The cellulose added to the incubation units was suspended in 0.5 mL aqueous solution. All substrates were injected into their incubation units at several different points in the sample. For each of the soil-substrate combinations, an identical unit that contained additional NAPL dissolved in dichloromethane was included. The quantity of total C added, as non-NAPL, substrate-C, to each soil incubation unit was 6.3 mg.

NAPLs

The soils were enriched with NAPL as described in chapter 5 (objective 2). Approximately twice the amount of NAPL present in each soil was added to each soil, in 5 mL dichloromethane. The non-NAPL amended treatments did not receive any solvent.

Nutrient Amendment

In all incubations, the soils were supplemented with $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4 as described in chapter 4.

Incubation

The experiment was set up as described in chapter 5. The experimental design was completely randomized with the addition of substrate to soil as the treatment. Thus, for the creosote-contaminated soil there were six treatments and for the oil-contaminated soil there were eight treatments. Each treatment was replicated three times. The duration of the incubation was 10 wk.

Carbon Fractionation

The carbon fractionation followed the same procedure described in chapter 5.

Statistics

One-way ANOVAs using SAS (Windows 6.10) PROC GLM were applied to the soil C fraction data sets. A Duncan's multiple range test was used to determine significant differences within these data sets ($p<0.05$). Non-parametric data analysis and a χ^2-test was used to determine if the $^{14}CO_2$ production curves were different. Non-parametric analysis and a χ^2-test was also applied to the C distributions in each soil to compare the distribution of ^{14}C remaining in a treatment unit at the end of 10 weeks with its (+ NAPL) counterpart. Due to the small number of data points collected to describe CO_2 evolution, this data set was not fit against a kinetic model.

Results

The cumulative $^{14}CO_2$ evolution from the non-NAPL amended treatments was significantly and substantially greater than the +NAPL plus cellulose treatments in both soils and than the plus NAPL plus octadecane treatment in the creosote-contaminated soil (Fig. 6-1). The evolution of $^{14}CO_2$ from the glucose-amended soils was also significantly greater in the +NAPL treatments than in the non-NAPL amended treatments, though the disparity was not as great (Fig. 6-2). The evolution of $^{14}CO_2$ from the acetate-amended and the pyrene-amended treatments of the oil-contaminated soil did not differ significantly with the additional NAPL. Predictably, the most water soluble substrates, glucose and acetate, have very little differences in their cumulative CO_2 production when compared with their additional NAPL counterparts.

The $^{14}CO_2$ production curves do show some differences. The curves for the glucose-amended treatments for both soils (Fig. 6-2) are quite different between no additional NAPL and +NAPL, based on non-parametric statistical comparison tests. The cumulative CO_2 production curves for the acetate + and - NAPL treatments in the oil-contaminated soil were different in magnitude, but followed an almost identical pattern, the no-additional NAPL treatment consistently slightly higher than the additional NAPL treatment (Fig. 6-2).

The CO_2 production curves for the cellulose-amended treatments did vary with the presence of additional NAPL in both soils (Fig. 6-1). The cellulose with -NAPL evolved consistently greater quantities of CO_2 than the cellulose with +NAPL in both soils. The

total $^{14}CO_2$ evolved from the -NAPL treatments was 4-times greater than the +NAPL treatment in the creosote-contaminated soil and 2-times greater than the +NAPL treatment in the oil-contaminated soil.

When the pyrene was spiked into the oil-contaminated soil (Fig. 6-1), the cumulative $^{14}CO_2$ evolved was very low (<2% of the applied ^{14}C), and the production curves for + and - additional NAPL were identical. Octadecane applied to the creosote-contaminated soil resulted in production curves in which the additional NAPL treatment had a first measurement slightly lower than the -NAPL treatment, and consistently produced slightly less CO_2.

The C distributions at the end of the experiment varied greatly with the different soils and substrates. When the distribution of ^{14}C among the WSC, HC, BC, DEOC, and HNC fractions are compared between +NAPL and -NAPL treatments, the distributions do not differ ($P<0.05$) for any substrate-soil combination. However, if we examine the measured values of individual fractions, differences do emerge (Fig. 6.3).

The total ^{14}C activity recovered was greater in the octadecane-amended soil for the no additional NAPL treatment than in the additional NAPL treatment. The total ^{14}C activity accounted for at the end of the experiment (sum of the activity measured as $^{14}CO_2$ and that measured in the C fractions, WSC, BC, HC, DEOC) was 99% in the additional NAPL treatment and 113% in the no additional NAPL treatment. This difference, counterbalanced by the difference in the ^{14}C activities of the DEO-C fractions, resulted in the same activity of ^{14}C calculated to have been retained in the humin.

The application of pyrene to the oil-contaminated soil with or without additional NAPL resulted in the same ($p<0.05$) ^{14}C activities measured in the WSC (9-14%), HC (15-18%), and BC (31%) fractions. Similar to the octadecane in the creosote-contaminated soil, significantly less ^{14}C was detected in the DEOC fraction of the additional NAPL treatment than in the no additional NAPL treatment. The activities of ^{14}C detected in the whole soils were the same in both treatments. The latter, combined with the difference in ^{14}C recovery in the DEOC results in the HNC fraction being calculated to be much larger in the additional NAPL treatment. The total ^{14}C accounted for was 91% in both treatments.

The ^{14}C from glucose was redistributed similarly in both soils, regardless of the presence or absence of additional NAPL. Of the applied ^{14}C, slightly more than 50% in the oil-contaminated soil, and slightly less than 50% in the creosote-contaminated soil was evolved as $^{14}CO_2$. Similar activities were recovered in the BC fraction: 8% in both NAPL treatments of the creosote-contaminated soil and 6% in both NAPL treatments of the oil-contaminated soil. Approximately 9% of the applied ^{14}C was recovered in the HC fraction of the creosote contaminated soil, 6-9% in the oil-contaminated soil. For both soils 3% of the applied ^{14}C activity was recovered in the WSC fraction and <1% in the DEO fraction. There is a slight difference in the HN fraction. In the creosote-contaminated soil 4% and 7% of the applied ^{14}C activity recovered in the + and - additional NAPL treatments, respectively and 12% and 18% recovered in the + and - additional NAPL treatments of the oil-contaminated soil.

In both soils amended with cellulose, the activity of ^{14}C measured in the whole soil was significantly lower and the total $^{14}CO_2$ evolved was significantly higher at the end of 10 wk in the no additional NAPL treatments than in the additional NAPL treatments. The

total activity accounted for at the end of the incubation was 64% and 73% for the no additional NAPL treatments of the creosote-contaminated and oil-contaminated soil respectively. The total ^{14}C recovered for the additional NAPL treatments were 90% and 94% for the creosote-contaminated and oil-contaminated soil respectively. No differences in ^{14}C activities were detected in the DEOC fractions of the additional NAPL treatments compared to the no additional NAPL treatments for both soils. The ^{14}C activities recovered in the remaining C fractions (BC, HC, WSC) were significantly lower in the no additional NAPL treatments for both soils.

Acetate was only applied to the oil-contaminated soil. No significant differences were measured in any of the C fractions between the no additional NAPL and additional NAPL treatments. Total ^{14}C activity recovered was ~100% of that applied. Of the ^{14}C applied, 42% (no additional NAPL) and 44% (additional NAPL) was evolved as $^{14}CO_2$, while 32% was recovered in the BMC, 7-10% in the HC, and 12-16% in the WSC. Less than 5% of the applied ^{14}C was recovered in the DEOC.

Discussion

The interpretation of the results of this experiment benefit by revisiting the findings presented in chapter 5, where it was determined there that results attributed to the addition of extra NAPL to these soils was actually due to the actions of the carrier solvent, dichloromethane. In discussing the results of this experiment, the NAPL-amended designations will still be used, however the reader is advised that the solvent is the modifying agent. The solvents in which the MOCs were carried into the soil were small quantities and highly volatile. There was no apparent redistribution of endogenous NAPL by the MOC-loading solvent observed in chapter 4, so possible NAPL-solvation by these solvents in this experiment is likely minimal.

The "cross-contamination" of the model organic contaminants into the non-type soils suppressed the evolution of $^{14}CO_2$ compared to previous studies. The total activity of $^{14}CO_2$ evolved from the oil-contaminated soil spiked with pyrene was much lower than that evolved from both the oil-contaminated soil spiked with octadecane (at 10 wk, 50% of the applied activity) and the pyrene spiked into the creosote-contaminated soil (at 10 wk, 60% of the applied activity) in chapter 4. It was similar to the total activity evolved from the creosote-contaminated soil amended with pyrene in the presence of dichloromethane-carried NAPL (<3%) in chapter 5. Similarly, the activity of $^{14}CO_2$ evolved from the creosote-contaminated soil spiked with octadecane was much lower than the activity evolved from either the creosote-contaminated soil spiked with pyrene or octadecane spiked into the oil-contaminated soil described in chapter 4. The activity evolved from the creosote-contaminated soil spiked with octadecane is lower too, than that evolved when octadecane was spiked into its "type" soil, the oil contaminated soil in the presence of additional NAPL (25% of the applied label evolved as $^{14}CO_2$; chapter 5). Both soil-MOC combinations in this experiment did exceed the activity of ^{14}C evolved from the creosote-contaminated soil spiked with pyrene in the presence of additional NAPL (0.6% of the activity applied; chapter 5).

Glucose is a commonly studied substrate in laboratory-based soil C studies that involve the addition of a new C source. The rich literature that has evolved around glucose proposes it to be a "priming agent" to both soil organic C mineralization (Wu *et*

al. 1993), and to contaminant mineralization (Shen and Bartha 1997). Conversely, glucose has also been observed to inhibit contaminant mineralization either by providing a simpler, more attractive substrate to the degrading microorganisms (Sato 1996; Sato and Lee 1996) or by promoting the growth of microorganisms that are unable to use contaminants as substrate but can out-compete those that do in the presence of a simple substrate (Guzev *et al.* 1997). Regardless of the organisms that were stimulated by applying glucose to these two soils, the result was an initially rapid and substantial metabolism of the carbohydrate tapering off after 30 d, so that half of the activity applied was mineralized to $^{14}CO_2$ in 10 wk. The remainder was found primarily in the HC and BC fractions or was not recovered. The persistence of the ^{14}C remaining in the soil beyond this point may be great, as the glucose-C has been incorporated into soil OM, particularly the HC fraction and is therefore part of the apparently unchanging but actually highly dynamic soil organic matter (Wu *et al.* 1993). If C utilization efficiency is calculated by Eq. 6-1, the CUE for glucose in both soils is close to 15%. This value is comparable to the CUE calculated for glucose in a non-contaminated clay soil after 66 d, reported by Chotte *et al.* (1998), 20%.

$$CUE = \left(\frac{C_{biomass}}{C_{biomass} + C_{CO_2}} \right) \times 100 \qquad \text{[Eq. 6-1]}$$

In this study, the presence of additional dichloromethane (+NAPL treatments) redistributed the NAPL in both contaminated soils and greatly diminished the activity of ^{14}C evolved as $^{14}CO_2$ from the cellulose-amended soils. Shen and Bartha (1997) found that 40% of the ^{14}C applied to a non-contaminated soil as cellulose had been mineralized within 30 d of incubation. Even in my no additional NAPL treatments, the activity of $^{14}CO_2$ evolved did not exceed 20% of the activity applied as cellulose after 10 wk of incubation. The contamination or the solvent perhaps, inhibited the cellulytic organisms present in these soils. It is also possible that cellulytic organisms were not present in these soils, or the substrate was not accessible in the presence of the NAPL or solvent. The NAPL may also be a sorptive phase for some of the capable microorganisms, so that rather than the MOC being inaccessible to the organism, the organism is unable to access the MOC. A final possibility is that the C contained in the NAPL is a preferred alternate substrate for the organisms that have survived in these contaminated soils for many years.

Applying acetate to the oil-contaminated soil in an attempt to provide a similar substrate to that released by the β-oxidation of straight-chain hydrocarbons resulted in less than half the applied label evolved as $^{14}CO_2$ during the 10-wk incubation, a value comparable to the amount of ^{14}C evolved in an earlier experiment in which octadecane was applied to the same soil (chapter 4). The organisms that oxidize acetate, as one would predict, do not differentiate between the sources of their substrate, a C_{18} alkane or its C_2 degradation product. The two other soil C fractions that were fates of the applied acetate-C in this experiment were BC and HC. Of the applied label, 32% was recovered as BC and 16% as HC. Less than 5% of the applied label was recovered in the DEO fraction. In the earlier experiment, with octadecane (chapter 4) less than 1% of the applied label was recovered in BC and 40% in HC after 10 wk. The ^{14}C recovery as WSC was small for both substrates (7% from the acetate, 4% from the octadecane in the earlier experiment). The relationship between the ^{14}C activities measured in BC and evolved as

$^{14}CO_2$ from the acetate-spiked oil-contaminated soil indicates that the acetate was directly metabolized by the soil microorganisms, an interpretation that is not so straightforward for the octadecane in the oil-contaminated soil (chapter 4).

The soils studied in this chapter both have recoverable contaminant residues that may exist as NAPLs and may be carriers and components of hazardous chemicals. The presence of NAPLs in a soil provides a phase into which organic contaminants having high K_{OW} values may partition (Efroymson and Alexander 1994, Fu and Alexander 1995). However, a significant characteristic of weathered contaminated soils is that the contaminant appears to be protected within pores and aggregates, separated from the microorganisms. Occluded within these pores, oxygen and water required for its metabolism may be limiting (Hatzinger and Alexander 1995). Other studies confirm that both soils studied here have been modified in this manner (chapter 4, chapter 5). From this, we have learned that the addition of extra NAPL to these soils is not necessary to improve the proximity of the MOC to the NAPL in order to enhance partitioning. The dichloromethane in which the NAPL was added to the soils was probably sufficient to redistribute the endogenous NAPL, and thus improve contact between MOC and a NAPL.

One observation from this experiment remains a problem: I am unable to explain the observation in both soils when spiked with the non-type MOC, of substantial quantities of ^{14}C detected in the biomass (42.5% in the creosote-contaminated soil, 31% in the oil-contaminated soil) with little evolved as $^{14}CO_2$ (<3% in both soils). The same values were observed in both the additional NAPL and no additional NAPL treatments of both soils. Chloroform fumigation may render non-biomass, contaminant-derived C more extractable to K_2SO_4, during the chloroform-fumigation extraction procedure, however two things persuade me that this is not the case. The first is that Joergensen *et al.* (1995) examined the chloroform fumigation-extraction method with respect to fuel oil-contaminated soils and found no evidence of enhanced contaminant extractability. The second is that measurement of a highly ^{14}C-enriched biomass without an appropriately large $^{14}CO_2$ loss has not been observed in any of the earlier experiments involving both soils and both model organic contaminants. A third possibility is that the MOCs sorbed physically to microbial cell walls, and were therefore counted as part of the biomass when the chloroform-fumigation lysed the microbial cells. If this occurred, the nature of the adaptation of suites of organisms to certain substrates would include selection based on physical characteristics as well as on biochemical capabilities.

To conclude, this experiment confirmed that the microorganisms present in these soils are capable of mineralizing different exotic C substrates. For all the substrate-soil combinations presented here, regardless of the solvent-mediated accessibility of a NAPL, transformation of the substrate into the BC and HC fractions was substantial. The concomitant evolution of $^{14}CO_2$ provides evidence that the transformations of the glucose, cellulose and acetate were biologically mediated. The accessibility of the NAPL may slightly decrease the mineralization of the non-hydrocarbon substrates examined, however their hydrophilic character suggests that the redistributed NAPLs act by: sequestering the microorganisms; physically separating the substrate, organism, and requirements for mineralization (nutrients, O_2), or; becoming alternative substrates themselves. Questions persist regarding the low level of $^{14}CO_2$ evolution and high degree of ^{14}C enrichment of the BC fraction when the PAH is cross-contaminated into the oil-contaminated soil and the

aliphatic hydrocarbon is cross-contaminated into the creosote-contaminated soil. This sequestration of the MOCs in the BC fraction without a corresponding respiration of this C is poorly understood.

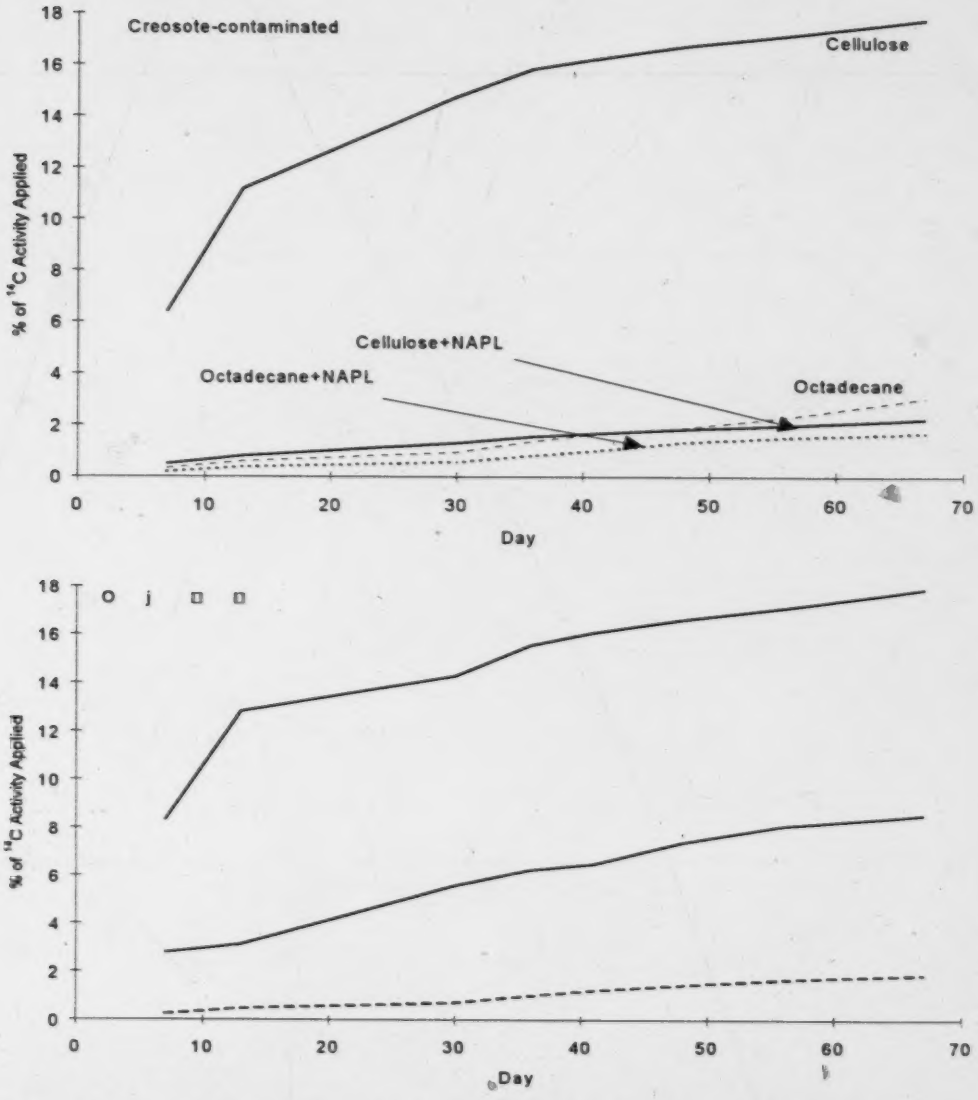

Figure 6-1. Cumulative $^{14}CO_2$ production curves for two soils incubated for 10 wk. A creosote-contaminated soil spiked with ^{14}C-octadecane or ^{14}C-cellulose in the presence or absence of additional NAPL and an oil-contaminated soil spiked with one of ^{14}C-pyrene or ^{14}C-cellulose in the presence or absence of additional NAPL. For the ^{14}C-cellulose in both soils, the curves for the treatments with additional NAPL are significantly different from those without extra NAPL. The curves for the soils with ^{14}C-pyrene or ^{14}C-octadecane do not differ between additional and no additional NAPL (χ^2 probabilities both >0.99).

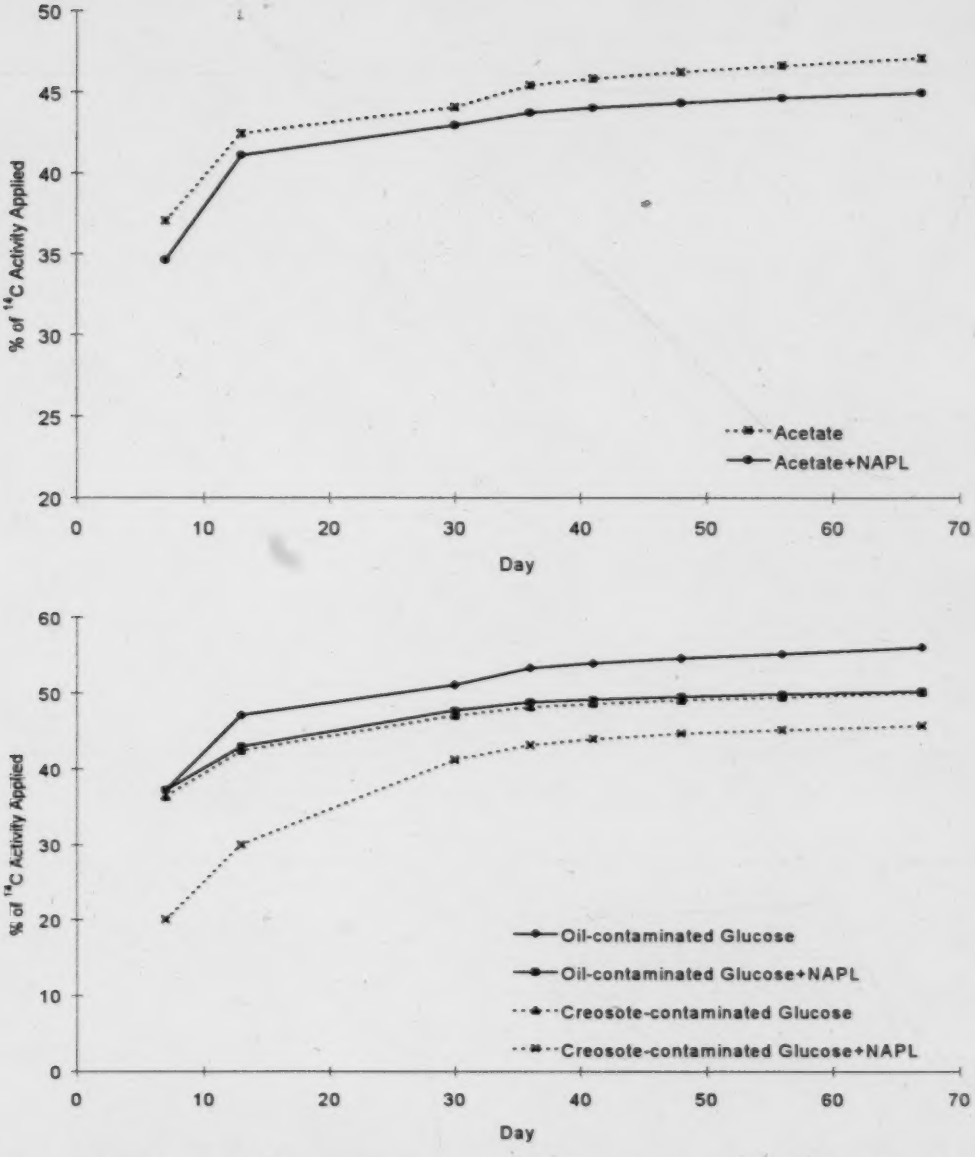

Figure 6-2. Cumulative $^{14}CO_2$ production curves for a soil amended with ^{14}C-acetate and for two soils amended with ^{14}C-glucose, both in the presence or absence of additional NAPL and incubated for 10 wk. The χ^2 probability that the two acetate treatment curves are the same >0.99. For glucose in each soil, the curves for the treatments without additional NAPL are significantly different from those with additional NAPL (χ^2 probability <0.25).

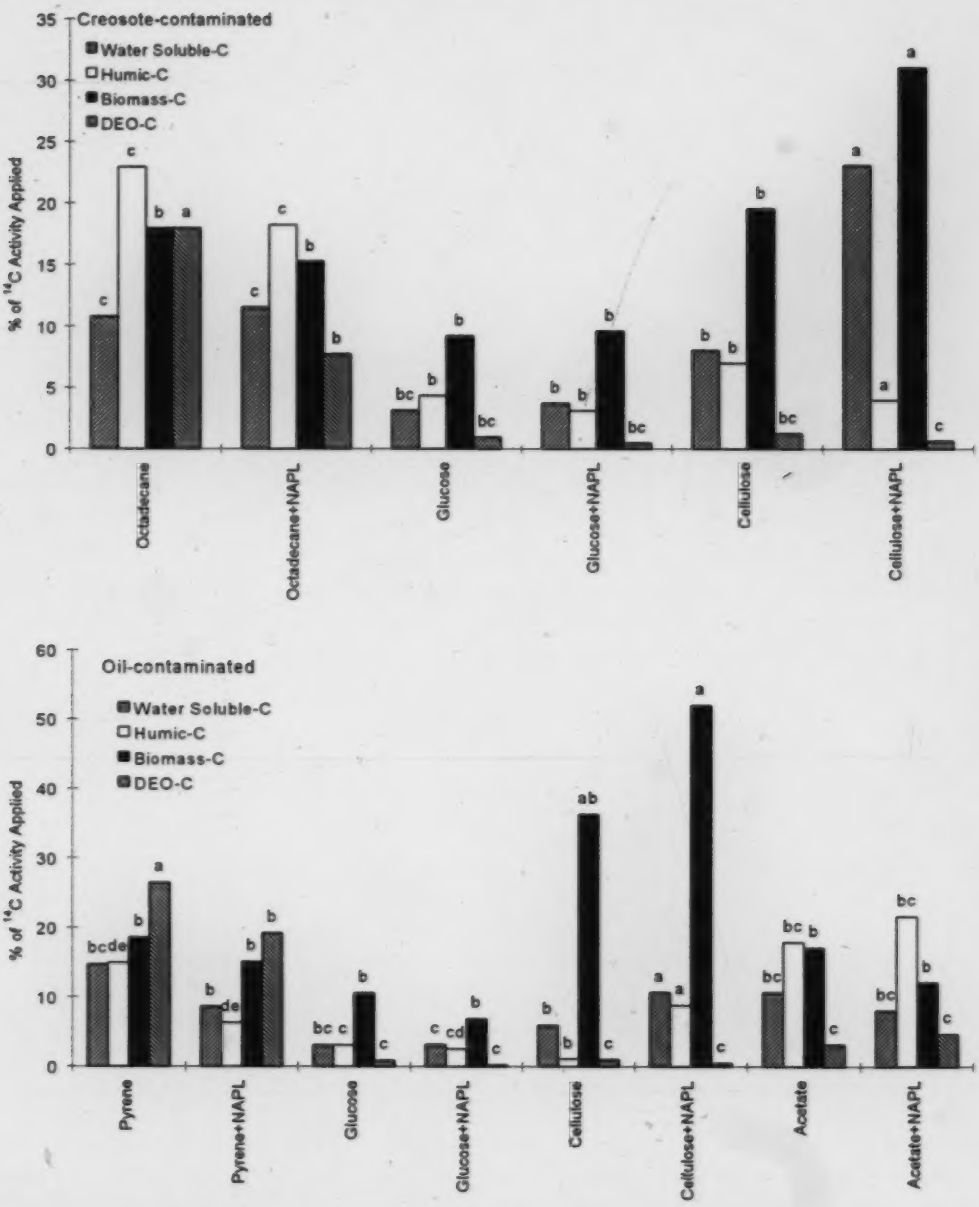

Figure 6-3. Soil C distributions after 10 wk incubation in a creosote-contaminated soil with ^{14}C-octadecane, ^{14}C-glucose, or ^{14}C-cellulose + or - extra NAPL and in an oil-contaminated soil with ^{14}C-pyrene, ^{14}C-glucose, ^{14}C-cellulose, or ^{14}C-acetate, + or - additional NAPL. C distributions, for the same substrate do not differ between no extra NAPL and extra NAPL. Comparisons shown are within fractions, amoung substrates. Bars with same letter are not statistically different ($P<0.05$).

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CHAPTER 7

SYNTHESIS

This thesis has extended the current state of knowledge of the fate of contaminant-C in weathered contaminated soils. As a consequence of some of these findings, more has been learned about the nature of weathered contaminated soils. This synthesis will examine first the fates of contaminant-C in these soils and then discuss the conjectures drawn regarding the soils themselves.

The fate of C in contaminated soils may be varied. In this thesis there is evidence that mineralization, humification, and incorporation into humin may all be fates of contaminant-C. I have also seen hints that contaminant-C compounds may be incorporated into plant tissues, a finding that merits further study. As well, the contaminant-C may persist in the NAPL phase associated with some types of organic contamination, either when both the substrate and the NAPL are freshly added to soils or when the residual NAPL in a weathered contaminated soil is redistributed within the soil by an appropriate solvent.

Mineralization of Contaminant-C in Contaminated Soils

If C mineralization to CO_2 is attributed to microbial respiration as they metabolize C substrates in soil, several implications may be drawn. First is that the evolution of 67% of the applied ^{14}C as CO_2 (from both soils), reported in chapter 4, where the MOCs were added to their type soils and incubated for a maximum of 12 mon, indicates the soil microorganisms had access to the MOCs, and were metabolizing them. Knowing that the soil organisms are capable of metabolizing these substrates, the decrease in the amount of CO_2 evolved from the soils in the presence of NAPL redistributed by dichloromethane indicates that the MOC was no longer accessible to the microorganisms. All of the incubation materials and methods were held constant to the first incubation, except the addition of the contaminant NAPL in dichloromethane and the mass of soil used (100 g in chapter 4; 50 in subsequent chapters). Therefore, it may be inferred that the decreased MOC availability was due to interactions of either the MOC or the microorganisms with the NAPL. The additional NAPL was not the critical factor in creating an accessible NAPL phase in these soils; the dichloromethane in which the NAPL was carried redistributed the endogenous NAPL in both soils to render the NAPL accessible. Since the two MOCs have high log K_{ow} values, it is most likely that partitioning of the MOCs into the NAPLs was the limiting reaction to MOC metabolism. Microorganisms are ubiquitous, and it is unlikely that they are all being occluded within the NAPL. Alternatively, the addition of dichloromethane may have killed part or all of the capable microorganisms in this soil. If this is the reason for the diminished CO_2 evolution, it appears to have selectively killed the hydrocarbon degraders and only partially killed others, because mineralization of non-hydrocarbon substrates was observed following solvent additions in chapter 6. An alternative hypothesis may be proposed: the NAPL may have formed an impenetrable barrier between the substrate and the microbe through which neither could pass. This hypothesis is rejected by findings presented in chapter 6, in which

compounds with low K_{ow} values (high water solubilities) were added to these soils in the presence of NAPLs and were metabolized to a similar extent as when they were added in the absence of NAPLs. For overcoming a physical barrier of NAPL to be the limiting step to microbial metabolism of the contaminant substrates, the contaminant must be the component that is specifically barred. This argument returns to the issue of high K_{ow} substrates partitioning into the NAPL, which I believe to be the most likely factor.

Transformations of Contaminant-C in Contaminated Soils

In considering the possible fates of contaminant-C in soils, only the previously discussed C mineralization was detected as a removal of ^{14}C from the system. In the oil-contaminated soil at 3 mon, humification accounted for one-third of the applied ^{14}C retained in the soil. This sequestration was apparently transient since by 12 mon, the humic-C had returned to the same level as week 1 (5.5%). A large amount of the applied ^{14}C, 40%, was determined to be bound in the humin. In both soils, "huminification" throughout the incubation may have played a significant role in retaining the MOC-C. By difference, approximately one-third of the applied ^{14}C was estimated to be associated with the non-extractable C (humin and other non-extractable C unique to contaminated soils) in the creosote-contaminated soil from months 6-10. While the quantities of C retained by the humin+humic fractions have a wide range of values, from 10-75% of the applied label, or may be transient, these results may provide promise to another soil science issue, discussed in the next section. The observed transience of the MOC-C enrichment in this combined C fraction suggests an alternate explanation. It is possible the the MOC was not transformed into the fractions, but was sorbed onto or into them. SOM is a known sorbent for organic macromolecules, and it would be rash to discount the possibility that some, if not all, of the MOC was sorbed. In light of the concomitant mineralization of ^{14}C, humic and humin ^{14}C enrichment, and the detection of organic forms of ^{15}N in these fractions, it is likely however, that at least part of the enrichment was microbially mediated.

The consistently low ^{14}C recoveries as biomass C in the soil systems that were spiked with MOC leads to another set of interpretations. There are three possible explanations for the low biomass C measurements. The first is that the MOCs were cometabolized, which means that they were being transformed gratuitously, with no benefit to the mediating organisms (Bossert and Compeau 1995). This still does not explain the production of $^{14}CO_2$, in the absence of ^{14}C-labeled biomass. A second possibility could simply be that the turnover time of the adapted population in the presence of an appropriate substrate is very quick. As well, there is a rich supply of other non-labeled C substrates in the soil that contribute to the biomass and may cycle preferentially through it. However, this too, cannot explain the production of $^{14}CO_2$ in the absence of ^{14}C-labeled biomass. A third possibility is that the transformations of the MOC were abiotic. I have rejected this possibility for the oil-contaminated soil on the basis of a concomitant enrichment of ^{15}N in the humic and biomass C fractions at 2 wk, 3 mon and 12 mon, as reported in Chapter 4.

The reverse situation was reported in Chapter 6, in which the biomass-C was highly enriched in the absence of a corresponding level of $^{14}CO_2$ evolution that was observed when the MOCs were cross-contaminated into the non-type soil in chapter 6. It

was proposed that the enrichment of the biomass with ^{14}C was due to the sorption of the MOC to microbial cell membranes. If one considers the paradox posed by the observations of biomass-mineralization correlations in chapters 4 and 6, it leads to some new proposals. If the MOC sorption to biomass, as proposed in chapter 6, reduces MOC bioavailability, it follows that the mineralization of MOC observed in chapter 4 indicates that the MOCs were not membrane-sorbed. Because the same two soils and MOCs were used, and only reversed between the two chapters, the sorption must have been through reactions specific to the membranes of the aliphatic-adapted suite of organisms and the aromatic compound and vice versa. This would mean that organisms evolve physically to keep their target substrate in a separate phase from themselves. It would also imply that sorption of the substrate to the microorganism blocks bioavailability.

This thesis began with a model of C transformations in soil, derived from the plant decomposition literature. We revisit a version of that model, now modified for contaminant-derived C and adjusted to accommodate the findings of this thesis (Fig. 7-1).

Some lower molecular weight hydrocarbons may be volatilized from the soil system. Non-volatile petroleum-derived contaminants can be separated into two fractions: degradable and slowly degradable. Components of either one of these fractions may undergo abiotic partitioning to SOM or soil minerals. This classification can be modified to enhance its pertinence to this thesis: the two fractions may be degradable or slowly degradable by virtue of their being accessible to microorganisms. Otherwise degradable components that partition into SOM or the endogenous NAPL phase, or are occluded within aggregates may become unavailable, and hence, behave as a slowly degradable component. However, the materials that are not protected, or that get exposed through tillage, freeze-thaw processes, faunal activity, *etc.*, must begin cycling through the SOM by being modified by the biomass (BC). The BC in the center of the diagram is distinguished here from the stored microbial biomass (B) component, as we perceive the fraction designated "B" to be for compounds which are in the biomass. "BC" is intended to describe the modification of the MOCs studied as they enter the biomass and are transformed into the other fractions. Due to the observation (chapter 4) that the enrichment of the HC may be transient, the humic materials (as described in this thesis) were placed into the labile OM component. The more persistent OM component is dominated by the humin fraction. This very stable OM component is likely the site of potential contaminant-SOM sorption reactions for which the desorption reaction may be slow. The kinetic parameter k^* is included to describe the rate of C mineralization. One should be aware that this rate constant will be specific to a kinetic model for C mineralization (*i.e.*, exponential, logistic, Gompertz), and therefore the appropriate model should be inserted. For the kinetic coefficients defined for the model, our data would predict that $K_B > K_A$, K_L, $> K_R > K_{HN}$. K_R becomes the rate of release of substrate from the poorly available component. The proportions described by the model would, from our data, be dependent on the availability of partitioning media for specific compounds. In the absence of readily accessible NAPL, P_B would apparently be small, while P_H and P_a would be larger. The loss of volatile hydrocarbons to the vapour phase is described by Henry's law constant (K_H). This constant may be calculated by dividing the vapour pressure of a pure compound by its solubility in water; therefore losses require that the compound have both a low solubility and a high vapour pressure. Therefore, compounds that are insoluble

in water are more likely to enter the vapour phase than are those that have only a high vapour pressure.

Applications of Contaminant-C Transformations in Contaminated Soils

A scientist studying greenhouse gases and C sequestration early in this program pointed out a problem in the traditional bioremediation studies. Dr. Cesar Izzaraulde pointed out the conflict between his research concerns and the traditional definition of successful bioremediation, complete oxidation of the contaminant to CO_2 (Izzaraulde 1996). Both fields have contributed to the understanding of global C dynamics. Atlas and Cerniglia (1995) hint that in the future, bioremediation may play a small role in solving C problems on a global scale, which may include removal of greenhouse gases from the atmosphere. The corollary is then that C may be sequestered in treated soils. Sequestration may be via transformation of contaminant into OM or via sorption into a partitioning media. This is a reaction often referred to as the "aging of a contaminant" in soil (Chung and Alexander 1998). I propose that the development of contaminant attenuation mechanisms that result in the sequestration of the contaminant-C in soil is not only advisable for reducing greenhouse gas emissions from soil, but also may contribute to increasing or maintaining the SOM levels in our soils.

The mechanisms credited with promoting C sequestration are similar to those described in this thesis for transforming C in contaminated soils: enhanced humification, sorption to SOM, and sorption to soil components. The results of the first incubation experiment (chapter 3) in which 4 contaminated soils were passively incubated for 10 wk were the first indication that the loss of C from DEO was not wholly due to mineralization of the contaminants. This discrepancy prompted the use of radiolabeled MOCs in a series of subsequent incubations. Evidence for the potential of humification as a mechanism of C sequestration and bioremediation was clearly shown in the experiments in which the two soils primarily studied were radiolabeled with MOCs: 12-mon incubation (maximum HC enrichment from MOC = 32%); MOC added to soils with extra NAPL and solvent (9%) and; MOC cross-contaminated into non-type soil (23%). Revisiting the general equation for organic chemical biodegradation (Atlas and Cerniglia 1995; Bossert and Compeau 1995) can provide a framework for estimating the potential C sequestration in a contaminated system.

$$Organic\ Contaminant \xrightarrow{microorganisms} CO_2 + H_2O + biomass(+partially\ oxidized\ metabolites)$$

The mean starting quantities of contaminant-derived C in the two soils studied in this thesis were 1.55% (oil-contaminated soil) and 0.92% (creosote contaminated soil). If it is imagined that a depth of 15 cm of soil were applied to the surface of a 1 ha field and managed such that 50% of the contaminant-C was retained in the soil as biomass and SOM components (McGill 1975), the quantity of C sequestered as SOM would be (assumptions: mass of a hectare furrow slice is 2 000 000 kg, and that SOM is ~58% C):

$$2000000\ kg\ soil \times 1.8\%C \times 50\%sequestered = 15500\ kg\ C\ stored\ ha^{-1}$$

$$15500\ kg\ C \times 1.72 = 26660\ kg\ SOM$$

and,

$$2000000\ kg\ soil \times 1.1\%C \times 50\%sequestered = 9200\ kg\ C\ stored\ ha^{-1}$$
$$11000\ kg\ C \times 1.72 = 15824\ kg\ SOM$$

Clearly, this could be an optimistic endpoint for contaminated soils. Garcia *et al.* (1992), applying municipal solid waste to soil and Biederbeck *et al.* (1993), applying enhanced oil-recovery sludges, both found increased SOM levels in the soil 3-5 yr after applying. Garcia *et al.* (1992) were attempting to rebuild eroded soils, and they attribute the OM enrichment to the increase in vegetation surviving on the eroded soil returning their residues seasonally. Biederbeck *et al.* (1993) were examining the potential for land application of sludges as a means of disposal, treatment, and as a possible ameliorative for weakly aggregated sandy soils. They measured increases of 2.01 kg C m^{-2} (equivalent to 35 Mg humus ha^{-1}) and attribute the increased levels to biodegradative transformations of the hydrocarbons facilitating incorporation into the SOM. The measured increase is similar to the quantities I calculated in my projection.

Integrating the knowledge generated by scientists working in the field of C sequestration with that generated by those working in the field of bioremediation can enrich both disciplines. Both also enhance the fundamental understanding of C chemistry with respect to soil-, substrate-, and organism-specific dynamics.

Although it did not follow the pattern of the incubation experiments presented in chapters 3-6, the plant-assisted bioremediation study presented in chapter 2 did yield information that was useful and an integral part of this thesis. 1) That one should consider the differences in gross analyses (*i.e.*, DEO) and compound-specific measurements, as the two measurements may lead to different findings. 2) The unique properties of weathered contaminated soils, mainly the predominance of heavy and complex residues in the DEO fraction hinder successful bioremediation. 3) That plants themselves may be a minor sink for some types of hydrocarbon contaminants.

One note to this thesis would be the inclusion of toxicity tests on these soils. This thesis has only considered the soil-contaminant-biological system. There has been no treatment of risk and hazard in this project. The transformations of contaminant-derived C in weathered contaminated soils were the primary focus of this thesis and the level of knowledge in that field has been advanced by this project.

Future work

This thesis has generated several ideas for future, follow-up projects. The most immediately relevent pursuits would be those that continue the thread of microbial access to substrate.

The first such question that I would like to see addressed is that of the physical location of residual aged hydrocarbons in soils. I speculate that these materials that were once distributed throughout the soil matrix are metabolized and weathered at the surfaces of stable aggregates. At these surfaces, microbes, O_2, and nutrients are readily available and metabolism may proceed until size, availability, or diffusivity excludes one of these components. At that time the residue is effectively "sequestered" within the aggregate.

The hypothesis that sorption of MOC to a non-adapted suite of microorganisms limits bioavailability could be pursued at the cellular level; a technique such as phospholipid-fatty acid methyl esterification applied to microbial cultures from soils adapted to different types of contamination may yield insights to the mechanism of substrate utilization, and thus accessibility.

The SOM-contaminant dynamics should be studied in more detail to clarify the association of MOC with humic-C and humin-C. Using chromatographic or fluorescence techniques to establish if the incorporated MOC-C has been transformed or is still intact could do this. Determining if a stable isotope (^{13}C) signature from the contaminant could be used to trace the contaminant dynamics in field-contaminated soils would be desirable because it would allow examination of the actual C dynamics. The practice of spiking a field contaminated soil with a traceable compound, as was done in this thesis, only allows one to guess at what transformations occurred in the original contaminant fraction over the past several decades based on what has been observed in a relatively short period of time.

Plant-assisted bioremediation experiments that quantify both gross measurements of contamination (such as DEO) as well as specific compounds should be conducted. Published literature in which DEO or total petroleum hydrocarbons were to assess phytoremediation success tends to be discouraging. Experiments in which specific compounds (either components of contamination or tracers) were tracked appear more successful. This paradox merits further study, as does the suggestion of plant uptake of oil-derived hydrocarbons.

A final goal of all future work should be to recognize that even though the characteristics of hydrocarbon-contamination may differ between sites, that the findings from all fields of C dynamics are relevant. This thesis has used bioremediation as a model system, however several decades of research into microbial metabolism, SOM research, soil physics, soil chemistry, agronomy, and engineering have stimulated much of the thought throughout its progress.

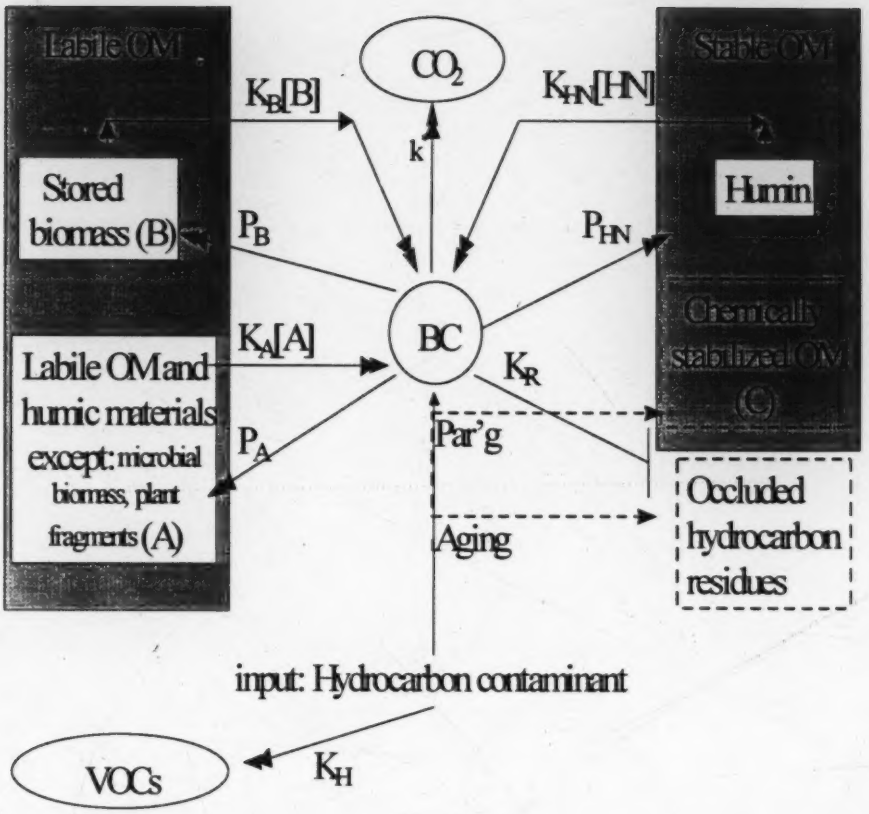

Figure 7-1. Model of fate of contaminant-derived C in soils (modified from Sallih and Pansu 1993) K_L, K_R = kinetic coefficients of decay in compartments V_L and V_R; K_H = Henry's constant; P_A, P_B, P_{HN} = input proportions into A, B, HN; k^* = rate of C mineralization. "Par'g" = partitioning.

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APPENDIX A:

Selected formulae used in this thesis.

$$gDBOg^{-1}soil = \left[\left(\frac{residue\,(g)}{soil\,extracted\,(g)}\right) \times \left(\frac{total\,extract\,volume\,(mL)}{volume\,of\,extract\,evaporated\,to\,residue\,(mL)}\right)\right] - Blank\,(g)$$

Chapter 2

Water stable aggregates in each sieve size fraction:

$$WSA_i = \left(\frac{\left(w_{2_i} - w_{3_i}\right)}{\left(\frac{w_1}{1+w_c}\right) - w_{3_i}}\right)$$

where: i = 1, 2, 3,…,n and corresponds to each size fraction

w_1 = starting mass of soil (oven-dry basis)

w_2 = oven-dry mass of aggregates collected in a size fraction

w_3 = sand correction (oven-dry mass of particles remaining on sieve after dispersion with sodium hexametaphosphate)

Mean weight diameter (mm):

$$MWD = \sum_{i=1}^{n} x_i WSA_i$$

where: i = 1,2,3,…,n and corresponds to each size fraction

x_i = the mean diameter of each size fraction

WSA_i is described in the previous calculation

Chapters 4-6

^{14}C activity in soil extracts:

$$Bq\,g^{-1}soil = \left(\frac{dpm \times \frac{1Bq}{60dpm}}{extracted\,soil\,(g)}\right) \times \left(\frac{extract\,counted\,(mL)}{total\,extract\,collected\,(mL)}\right)$$

Biomass C:

$$C_{Biomass} = \frac{\left(C_{fumigated} - C_{control}\right)}{k_{ec}}$$

where: $C_{fumigated}$= the quantity of C (or activity of ^{14}C) measured g^{-1} chloroform-fumigated soil

$C_{control}$= the quantity of C (or activity of ^{14}C) measured g^{-1} non-fumigated control soil k_{ec}= Biomass C extraction efficiency coefficient (0.25)

APPENDIX B:

^{14}C counting statistics

	Background dpm			Sample dpm
Solution	mean	range	standard deviation	minimum[1]
Water	18.7	17.0-22.2	1.22	18.3[3]
NaOH (0.5 M)	17.6	12.3-21.7	2.49	18.5[3]
K_2SO_4 (0.5 M)	31.4	26.8-36.6	4.00	35.1[3]
CH_2Cl_2	61.0	39.5-87.1	19.2	476.62[3]
KOH[2]	27.9	21.6-34.0	1.59	45.2[4]

[1]Values of zero ^{14}C enrichment were determined in some replicates for WSC, HC, and BC for all experiments using radiolabeled compounds; minimum values reported are the minimum for all experiments.

[2]Value reported for background was collected over 7 d; value reported for sample minimum was collected over 2 d.

[3]These minimum values were detected in the raw data collected from the experiment reported in chapter 4

[4]This minimum value was detected in the raw data collected from the experiment reported in chapter 5 (objective 1, creosote-contaminated soil (NAPL+^{14}C)).